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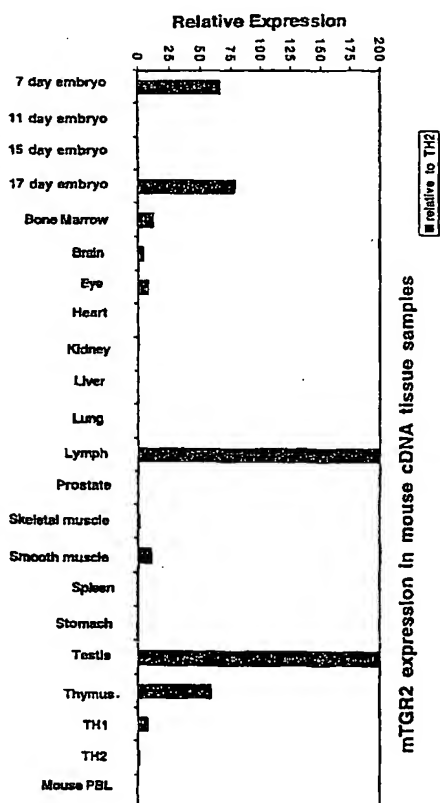
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[Continued on next page]

(54) Title: **NOVEL RECEPTORS**



(57) Abstract: The invention provides isolated nucleic acid and amino acid sequences of novel receptors, antibodies to such receptors, methods of detecting such nucleic acids and receptors, and methods of screening for modulators of the receptors.

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## NOVEL RECEPTORS

### CROSS-REFERENCES TO RELATED APPLICATIONS

[01] This application claims the benefit of U.S. provisional application no.  
5 60/302,800, filed July 3, 2001, which is herein incorporated by reference.

### BACKGROUND OF THE INVENTION

[02] The invention provides isolated nucleic acid and amino acid sequences of six  
novel receptors, antibodies to such receptors, methods of detecting such nucleic acids and  
10 receptors, and methods of screening for modulators of G-protein coupled receptors.

[03] G-protein coupled receptors are cell surface receptors that indirectly transduce  
extracellular signals to downstream effectors, which can be intracellular signaling proteins,  
enzymes, or channels, and changes in the activity of these effectors then mediate subsequent  
cellular events. The interaction between the receptor and the downstream effector is  
15 mediated by a G-protein, a heterotrimeric protein that binds GTP. G-protein coupled  
receptors ("GPCRs") typically have seven transmembrane regions, along with an  
extracellular domain and a cytoplasmic tail at the C-terminus. These receptors form a large  
superfamily of related receptors molecules that play a key role in many signaling processes,  
such as sensory and hormonal signal transduction. For example, a large family of olfactory  
20 GPCRs has been identified (*see, e.g.,* Buck & Axel, *Cell* 65:175-187 (1991)). The further  
identification of GPCRs is important for understanding the normal process of signal  
transduction and as well as its involvement in pathologic processes. For example, GPCRs  
can be used for disease diagnosis as well as for drug discovery. Further identification of  
novel GPCRs is therefore of great interest.

25

### SUMMARY OF THE INVENTION

[04] The present invention thus provides for the first time nucleic acids encoding  
novel G-protein coupled receptors, methods of detecting such receptors and the nucleic acids  
encoding them, methods of identifying modulators of such receptors, and methods of  
30 diagnosing and treating disease states associated with the receptors or mutants thereof.

[05] In one aspect, the invention provides an isolated nucleic acid encoding a  
polypeptide comprising at least 70% amino acid identity to an amino acid sequence of SEQ



ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12. In some embodiments, the nucleic acid encodes a polypeptide comprising at least 80%, often 90%, amino acid identity to an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12. In other embodiments, the  
5 nucleic acid comprises the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11.

[06] In another embodiment, the nucleic acid encodes a polypeptide that specifically binds to polyclonal antibodies generated against an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.  
10 The polypeptide can have G-protein coupled receptor activity. In some embodiments, the polypeptide comprises an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

[07] In other embodiments, the nucleic acid is amplified by primers that specifically hybridize under stringent hybridization conditions to SEQ ID NO:1, SEQ ID  
15 NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11.

[08] In another aspect, the present invention provides an isolated nucleic acid encoding a G-protein coupled receptor polypeptide, wherein the nucleic acid specifically hybridizes under stringent hybridization conditions to a nucleic acid comprising a nucleotide  
20 sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5; SEQ ID NO:7, SEQ ID NO:9; or SEQ ID NO:11.

[09] In another aspect, the invention provides an isolated nucleic acid encoding a polypeptide comprising at least 70% amino acid identity to a polypeptide having an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID  
25 NO:10, or SEQ ID NO:12, wherein the nucleic acid selectively hybridizes under moderately stringent hybridization conditions to a nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11. In some embodiments, the nucleic acid selectively hybridizes under stringent conditions to a nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID  
NO:11.

30 [10] In another aspect, the invention provides an isolated nucleic acid encoding a polypeptide comprising at least 15, often 20 contiguous amino acids of an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

[11] In another aspect, the invention provides an isolated polypeptide comprising at least 70% amino acid sequence identity to an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12. In some embodiments, the polypeptide comprises 80%, or 90% identity to the amino acid sequence.

5 In other embodiment, the polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10; or SEQ ID NO:12.

[12] In another embodiment, the polypeptide that specifically binds to polyclonal antibodies generated against an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

[13] In another embodiment, the polypeptide that has G-protein coupled receptor activity.

[14] In some embodiment, the polypeptide comprises at least 15, often 20 contiguous amino acids of an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

[15] In another aspect, the present invention provides an antibody that binds to a polypeptide of the invention.

[16] In another aspect, the present invention provides expression vectors comprising the nucleic acids of the invention, and host cells comprising the expression vectors.

[17] In another aspect, the invention provides a method for identifying a compound that modulates signal transduction, the method comprising: (i) contacting the compound with a polypeptide comprising at least 70% amino acid sequence identity to an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12; and (ii) determining the functional effect of the compound upon the polypeptide. In some embodiments, the polypeptide comprises at least 80% or at least 90% identity to the amino acid sequence.

[18] Alternatively, the invention provides a method of identifying a compound that modulates signal transduction, the method comprising: (i) contacting the compound with a polypeptide comprising at least 15, often 20 contiguous amino acids of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.; and (ii) determining the functional effect of the compound upon the polypeptide.

[19] In one embodiment, the polypeptide comprises the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

[20] In one embodiment, the polypeptide has G-protein coupled receptor activity.  
5 In another embodiment, the polypeptide is linked to a solid phase. In an additional embodiment, the polypeptide is covalently linked to a solid phase.

[21] In one embodiment, the functional effect is determined by measuring changes in intracellular cAMP, IP<sub>3</sub>, or Ca<sup>2+</sup>. In another embodiment, the functional effect is a chemical effect or a physical effect. In another embodiment, the functional effect is  
10 determined by measuring binding of the compound to the polypeptide.

[22] In one embodiment, the polypeptide is recombinant.

[23] In one embodiment, the polypeptide is expressed in a cell or cell membrane. In another embodiment, the cell is a eukaryotic cell. In another embodiment, the cell is a brain, adipose, kidney, skeletal muscle, pancreas, lung, immune system cell, small intestine,  
15 liver, stomach, colon, prostate, or placenta.

[24] In another aspect, the invention provides a method of identifying a mammal having a TGR-associated disorder, the method comprising detecting a nucleic acid molecule that comprises greater than 70% nucleic acid sequence identity to a sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11 in a  
20 sample from the mammal, wherein abnormal expression of the nucleic acid molecule in the sample indicates that the mammal has a TGR-associated disorder. In one embodiment, the nucleic acid molecule comprises the sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11.

[25] Additionally, the invention provides a method of identifying a mammal having  
25 a TGR-associated disorder, the method comprising detecting a nucleic acid molecule that comprises at least 15, often 20, contiguous nucleotides of a nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11 in a sample from the mammal, wherein abnormal expression of the nucleic acid molecule in the sample indicates that the mammal has a TGR-associated disorder.

[26] The invention also provides a method of identifying a mammal having a TGR-associated disorder, the method comprising detecting a polypeptide that comprises greater than 70% amino acid sequence identity to an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12 in a sample from the  
30 mammal, wherein abnormal expression of the polypeptide in the sample indicates that the

mammal has a TGR-associated disorder. In one embodiment, the polypeptide comprises the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

[27] In addition, the invention provides a method of identifying a mammal having a TGR-associated disorder, the method comprising detecting a polypeptide that comprises at least 15, often 20, contiguous amino acids of an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12 in a sample from the mammal, wherein abnormal expression of the polypeptide in the sample indicates that the mammal has a TGR-associated disorder.

[28] In another aspect, the invention provides a method of detecting the presence of a TGR-GPCR nucleic acid or polypeptide in human tissue, the method comprising: (i) isolating a biological sample; (ii) contacting the biological sample with a TGR-GPCR-specific reagent that selectively associates with an TGR-GPCR nucleic acid or polypeptide; and, (iii) detecting the level of TGR-GPCR-specific reagent that selectively associates with the sample.

[29] In one embodiment, the TGR-GPCR-specific reagent includes, but is not limited to, antibodies, oligonucleotide primers, and nucleic acid probes.

[30] In another aspect, the present invention provides a method of treating a patient with a disease or condition associated with a GPCR activity, comprising administering to the patient a modulator of a GPCR sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12. In one embodiment, the patient who has, or is at risk for, asthma, is administered a TGR2 modulator. In another embodiment, a patient who has, or is at risk for, multiple sclerosis is administered a TGR343 modulator. In another embodiment, a patient who has, or is at risk for, kidney disease is administered a TGR164 modulator.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[31] Figure 1 shows TGR2 expression in a tissue panel. Expression was analyzed by quantitative PCR.

[32] Figure 2 shows TGR2 expression in a model of asthma. Asthma was induced using ovalbumin (Djukanovic, *Clin. Exp. Allergy Jun;30 Suppl 1*:46-50, review). Tissues were collected and analyzed by RT-PCR. BAL, bronchoalveolar lavage.

[33] Figure 3 shows TGR343 expression in mouse and human tissue samples. Expression was determined by quantitative RT-PCR (QPCR).

[34] Figure 4 shows expression of TGR343 in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis.

#### DETAILED DESCRIPTION OF THE INVENTION

5 [35] The present invention provides for the first time nucleic acids encoding six novel G protein coupled receptors. The six nucleic acids and the receptors that they encode are referred to individually as TGR2, TGR38, TGR118, TGR164, TGR343, and TGR358. These GPCRs are components of signal transduction pathways in a variety of cells. These nucleic acids and the encoded receptors provide, *inter alia*, valuable probes for the  
10 identification of particular cell types, as certain of them show specific patterns of expression, for the isolation of specific modulators of GPCR activity in different cell types, for use as genetic markers, as the chromosomal location of many of them is known, and for the identification of mutations associated with diseases resulting from GPCR inactivation in particular cell types. Nucleic acids encoding the GPCRs of the invention can be identified  
15 using techniques such as reverse transcription and amplification of mRNA, isolation of total RNA or poly A+ RNA, northern blotting, dot blotting, in situ hybridization, RNase protection, S1 digestion, probing DNA microchip arrays, and the like.

[36] Chromosome localization of several of the genes has been determined, *e.g.*, TGR2 is localized to human chromosome 11q13. These GPCR genes can be used to identify  
20 diseases, mutations, and traits caused by and associated with the GPCRs.

[37] Various aspects of the cell-type specific expression of the present GPCRs has been determined and are as follows: human TGR2 is expressed in the immune system, brain, adipose, kidney, skeletal muscle, smooth muscle, testis, and placenta; human TGR38 is expressed in adipose, placenta, kidney, pancreas, spleen, skin, colon, skeletal muscle, liver,  
25 and brain; human TGR118 is expressed in brain and peripheral blood lymphocytes (PBLs); human TGR164 is expressed in kidney, brain, adipose, pancreas, lung, and colon; human TGR343 is expressed in the immune system, and in the hypothalamus, adipose, liver, kidney, placenta, lung, colon, thymus, spleen, breast, and skin; and human TGR358 is expressed in brain, adipose, skeletal muscle, heart, placenta, colon, prostate, stomach, small intestine,  
30 kidney, lung, thymus, and spleen. Such tissue specific expression indicates that the present GPCRs can be used to specifically modulate GPCR activity in particular cell types. In addition, certain diseases or conditions, or a propensity for the diseases or conditions, may be detected by detecting mutations in particular GPCRs, as described *infra*.

[38] The isolation of novel GPCRs provides a means for assaying for and identifying modulators of G-protein coupled receptor signal transduction, *e.g.*, activators, inhibitors, stimulators, enhancers, agonists, and antagonists. Such modulators of signal transduction are useful for pharmacological modulation of signaling pathways, *e.g.*, in cells and tissues such as brain, hypothalamus, adipose, kidney, pancreas, skeletal muscle, placenta, lung, colon, liver, spleen, thymus, prostate, stomach, and small intestine. Such activators and inhibitors identified using GPCRs can also be used to further study signal transduction. Thus, the invention provides assays for signal transduction modulation, where the GPCRs act as direct or indirect reporter molecules for the effect of modulators on signal transduction.

5

10 GPCRs can be used in assays *in vitro*, *ex vivo*, and *in vivo*, *e.g.*, to measure changes in transcriptional activation of GPCRs; ligand binding; phosphorylation and dephosphorylation; GPCR binding to G-proteins; G-protein activation; regulatory molecule binding; voltage, membrane potential, and conductance changes; ion flux; changes in intracellular second messengers such as cAMP and inositol triphosphate; changes in intracellular calcium levels; and neurotransmitter release.

15

[39] Methods of assaying for modulators of signal transduction include *in vitro* ligand binding assays using the GPCRs, portions thereof such as the extracellular domain, or chimeric proteins comprising one or more domains of a GPCR, oocyte GPCR expression or tissue culture cell GPCR expression, either naturally occurring or recombinant; membrane expression of a GPCR, either naturally occurring or recombinant; tissue expression of a GPCR; expression of a GPCR in a transgenic animal, *etc.*

20

[40] Functionally, the GPCRs represent a seven transmembrane G-protein coupled receptor of the G-protein coupled receptor family, which interact with a G protein to mediate signal transduction (*see, e.g.*, Fong, *Cell Signal* 8:217 (1996); Baldwin, *Curr. Opin. Cell Biol.* 6:180 (1994)).

25

[41] Related GPCR genes from other species should share at least about 70%, 80%, 90%, or greater, amino acid identity over an amino acid region at least about 25 amino acids in length, optionally 50 to 100 amino acids in length.

[42] Specific regions of the GPCR nucleotide and amino acid sequences may be used to identify polymorphic variants, interspecies homologs, and alleles of GPCRs. This identification can be made *in vitro*, *e.g.*, under stringent hybridization conditions or PCR (using primers that hybridize to SEQ ID NOS:1, 3, 5, 7, 9, or 11) and sequencing, or by using the sequence information in a computer system for comparison with other nucleotide sequences. Typically, identification of polymorphic variants and alleles of a GPCR is made

30

by comparing an amino acid sequence of about 25 amino acids or more, *e.g.*, 50-100 amino acids. Amino acid identity of approximately at least 70% or above, optionally 75%, 80%, 85% or 90-95% or above typically demonstrates that a protein is a polymorphic variant, interspecies homolog, or allele of a GPCR. Sequence comparison is performed using the  
5 BLAST and BLAST 2.0 sequence comparison algorithms with default parameters, discussed below. Antibodies that bind specifically to a GPCR or a conserved region thereof can also be used to identify alleles, interspecies homologs, and polymorphic variants. The polymorphic variants, alleles and interspecies homologs are expected to retain the seven transmembrane structure of a G-protein coupled receptor.

10 [43] GPCR nucleotide and amino acid sequence information may also be used to construct models of GPCRs in a computer system. These models are subsequently used to identify compounds that can activate or inhibit GPCRs. Such compounds that modulate the activity of a GPCR can be used, *e.g.*, to investigate the role of GPCRs in signal transduction.

## 15 Definitions

[44] "GPCR," "TGR2, TGR38, TGR118, TGR164, TGR343, and TGR358," and "TGR-GPCR" all refer to novel G-protein coupled receptors, the genes for most of which have been mapped to particular chromosomes and which are expressed in particular cell types. The GPCRs of the invention have seven transmembrane regions and have "G-protein  
20 coupled receptor activity," *e.g.*, they bind to G-proteins in response to extracellular stimuli and promote production of second messengers such as IP<sub>3</sub>, cAMP, and Ca<sup>2+</sup> via stimulation of downstream effectors such as phospholipase C and adenylate cyclase (for a description of the structure and function of GPCRs, *see, e.g.*, Fong, *supra*, and Baldwin, *supra*).

[45] Topologically, GPCRs have an N-terminal "extracellular domain," a  
25 "transmembrane domain" comprising seven transmembrane regions and corresponding cytoplasmic and extracellular loops, and a C-terminal "cytoplasmic domain" (*see, e.g.*, Buck & Axel, *Cell* 65:175-187 (1991)). These domains can be structurally identified using methods known to those of skill in the art, such as sequence analysis programs that identify hydrophobic and hydrophilic domains (*see, e.g.*, Kyte & Doolittle, *J. Mol. Biol.* 157:105-132  
30 (1982)). Such domains are useful for making chimeric proteins and for *in vitro* assays of the invention.

[46] "Extracellular domain" therefore refers to the domain of a GPCR that protrudes from the cellular membrane and often binds to an extracellular ligand. This domain is often useful for *in vitro* ligand binding assays, both soluble and solid phase.

[47] "Transmembrane domain," comprises seven transmembrane regions plus the corresponding cytoplasmic and extracellular loops. Certain regions of the transmembrane domain can also be involved in ligand binding.

[48] "Cytoplasmic domain" refers to the domain of a GPCR that protrudes into the cytoplasm after the seventh transmembrane region and continues to the C-terminus of the polypeptide.

[49] "GPCR activity" refers to the ability of a GPCR to transduce a signal. Such activity can be measured, *e.g.*, in a heterologous cell, by coupling a GPCR (or a chimeric GPCR) to a G-protein and a downstream effector such as PLC, and measuring increases in intracellular calcium (*see, e.g.*, Offermans & Simon, *J. Biol. Chem.* 270:15175-15180 (1995)). Receptor activity can be effectively measured by recording ligand-induced changes in  $[Ca^{2+}]_i$  using fluorescent  $Ca^{2+}$ -indicator dyes and fluorometric imaging.

[50] The terms "GPCR" and "TGR-2, -38, -118, -164, -343, and -358" therefore refer to nucleic acid and polypeptide polymorphic variants, alleles, mutants, and interspecies homologs and GPCR domains thereof that: (1) have an amino acid sequence that has greater than about 65% amino acid sequence identity, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater amino acid sequence identity, preferably over a region of over a region of at least about 25, 50, 100, 200, 500, 1000, or more amino acids, to a sequence of SEQ ID NO:2; 4, 6, 8, 10, or 12 over a window of about 25 amino acids, preferably 50-100 amino acids; (2) bind to antibodies raised against an immunogen comprising an amino acid sequence of SEQ ID NO:2, 4, 6, 8, 10, or 12 and conservatively modified variants thereof; (3) specifically hybridize (with a size of at least about 100, preferably at least about 500 or 1000 nucleotides) under stringent hybridization conditions to a sequence SEQ ID NO:1, 3, 5, 7, 9, or 11, and conservatively modified variants thereof; (4) have a nucleic acid sequence that has greater than about 95%, preferably greater than about 96%, 97%, 98%, 99%, or higher nucleotide sequence identity, preferably over a region of at least about 50, 100, 200, 500, 1000, or more nucleotides, to SEQ ID NOs: 1, 3, 5, 7, 9, or 11; (5) are amplified by primers that specifically hybridize under stringent conditions to SEQ ID NO: 1, 3, 5, 7, 9, or 11. This term also refers to a domain of a GPCR, as described above, or a fusion protein comprising a domain of a GPCR linked to a heterologous protein. A TGR-2, -38, -118, -164, -343, or -358 protein or domain typically comprises 10, 15, often 20, 25, or 30 or more contiguous amino acids of SEQ ID NO:2, 4, 6, 8, 10, or 12. A TGR-2, -38, -118, -164, -343, or -358 nucleic acid typically comprises at least 15, often 20, 25, 30, or 50 or more contiguous nucleotides of a sequence of SEQ ID NOs: 1, 3, 5, 7, 9, or 11. GPCR



polynucleotide or polypeptide sequence of the invention is typically from a mammal including, but not limited to, human, rat, mouse, hamster, cow, pig, horse, sheep, or any mammal. A "TGR-2, -38, -118, -164, -343, and -358 polynucleotide" and a "TGR- TGR-2, -38, -118, -164, -343, and -358 polypeptide," are both either naturally occurring or  
5 recombinant.

[51] A "full length" TGR-2, -38, -118, -164, -343, or -358 protein or nucleic acid refers to a polypeptide or polynucleotide sequence, or a variant thereof, that contains all of the elements normally contained in one or more naturally occurring, wild type TGR-2, -38, -118, -164, -343, or -358 polynucleotide or polypeptide sequences. It will be recognized,  
10 however, that derivatives, homologs, and fragments of TGR- TGR-2, -38, -118, -164, -343, or -358 can be readily used in the present invention.

[52] A "host cell" is a naturally occurring cell or a transformed cell that contains an expression vector and supports the replication or expression of the expression vector. Host cells may be cultured cells, explants, cells *in vivo*, and the like. Host cells may be  
15 prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells such as CHO, HeLa, and the like.

[53] "Biological sample" as used herein is a sample of biological tissue or fluid that contains nucleic acids or polypeptides of novel GPCRs. Such samples include, but are not limited to, tissue isolated from humans, mice, and rats,. Biological samples may also include  
20 sections of tissues such as frozen sections taken for histologic purposes. A biological sample is typically obtained from a eukaryotic organism, such as insects, protozoa, birds, fish, reptiles, and preferably a mammal such as rat, mouse, cow, dog, guinea pig, or rabbit, and most preferably a primate such as chimpanzees or humans. Preferred tissues typically depend on the known expression profile of the GPCR, and include *e.g.*, normal colon, spleen, kidney,  
25 liver, hypothalamus, adipose, or other tissues.

[54] The phrase "functional effects" in the context of assays for testing compounds that modulate GPCR-mediated signal transduction includes the determination of any parameter that is indirectly or directly under the influence of a GPCR, *e.g.*, a functional, physical, or chemical effect. It includes ligand binding, changes in ion flux, membrane  
30 potential, current flow, transcription, G-protein binding, gene amplification, expression in cancer cells, GPCR phosphorylation or dephosphorylation, signal transduction, receptor-ligand interactions, second messenger concentrations (*e.g.*, cAMP, cGMP, IP<sub>3</sub>, or intracellular Ca<sup>2+</sup>), *in vitro*, *in vivo*, and *ex vivo* and also includes other physiologic effects such increases or decreases of neurotransmitter or hormone release.

[55] By “determining the functional effect” is meant assays for a compound that increases or decreases a parameter that is indirectly or directly under the influence of a GPCR, *e.g.*, functional, physical and chemical effects. Such functional effects can be measured by any means known to those skilled in the art, *e.g.*, changes in spectroscopic characteristics (*e.g.*, fluorescence, absorbance, refractive index), hydrodynamic (*e.g.*, shape), chromatographic, or solubility properties, patch clamping, voltage-sensitive dyes, whole cell currents, radioisotope efflux, inducible markers, transcriptional activation of GPCRs; ligand binding assays; voltage, membrane potential and conductance changes; ion flux assays; changes in intracellular second messengers such as cAMP and inositol triphosphate (IP3); changes in intracellular calcium levels; neurotransmitter release, and the like.

[56] “Inhibitors,” “activators,” and “modulators” of GPCRs refer to inhibitory, activating, or modulating molecules identified using *in vitro* and *in vivo* assays for signal transduction, *e.g.*, ligands, agonists, antagonists, and their homologs and mimetics. Such modulating molecules, also referred to herein as compounds, include polypeptides, antibodies, amino acids, nucleotides, lipids, carbohydrates, or any organic or inorganic molecule. Inhibitors are compounds that, *e.g.*, bind to, partially or totally block stimulation, decrease, prevent, delay activation, inactivate, desensitize, or down regulate signal transduction, *e.g.*, antagonists. Activators are compounds that, *e.g.*, bind to, stimulate, increase, open, activate, facilitate, enhance activation, sensitize or up regulate signal transduction, *e.g.*, agonists. Modulators include compounds that, *e.g.*, alter the interaction of a polypeptide with: extracellular proteins that bind activators or inhibitors; G-proteins; G-protein *alpha*, *beta*, and *gamma* subunits; and kinases. Modulators also include genetically modified versions of GPCRs, *e.g.*, with altered activity, as well as naturally occurring and synthetic ligands, antagonists, agonists, antibodies, small chemical molecules and the like. Such assays for inhibitors and activators include, *e.g.*, expressing GPCRs *in vitro*, in cells, or cell membranes, applying putative modulator compounds, and then determining the functional effects on signal transduction, as described above.

[57] Samples or assays comprising GPCRs that are treated with a potential activator, inhibitor, or modulator are compared to control samples without the inhibitor, activator, or modulator to examine the extent of inhibition. Control samples (untreated with inhibitors) are assigned a relative GPCR activity value of 100%. Inhibition of a GPCR is achieved when the GPCR activity value relative to the control is about 80%, preferably 50%, more preferably 25-0%. Activation of a GPCR is achieved when the GPCR activity value relative to the control (untreated with activators) is 110%, more preferably 150%, more

preferably 200-500% (i.e., two to five fold higher relative to the control), more preferably 1000-3000% higher.

[58] The terms "isolated" "purified" or "biologically pure" refer to material that is substantially or essentially free from components which normally accompany it as found in its native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein that is the predominant species present in a preparation is substantially purified. In particular, an isolated GPCR nucleic acid is separated from open reading frames that flank the GPCR gene and encode proteins other than the GPCR. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

[59] "Biologically active" GPCR refers to a GPCR having signal transduction activity and G protein coupled receptor activity, as described above. .

[60] "Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

[61] Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzner *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

[62] The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding

naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

[63] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline,  $\gamma$ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an  $\alpha$  carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[64] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[65] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

[66] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration  
5 results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[67] The following eight groups each contain amino acids that are conservative  
10 substitutions for one another:

- 1) Alanine (A), Glycine (G);
  - 2) Aspartic acid (D), Glutamic acid (E);
  - 3) Asparagine (N), Glutamine (Q);
  - 4) Arginine (R), Lysine (K);
  - 15 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
  - 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
  - 7) Serine (S), Threonine (T); and
  - 8) Cysteine (C), Methionine (M)
- (see, e.g., Creighton, *Proteins* (1984)).

20 [68] Macromolecular structures such as polypeptide structures can be described in terms of various levels of organization. For a general discussion of this organization, see, e.g., Alberts *et al.*, *Molecular Biology of the Cell* (3<sup>rd</sup> ed., 1994) and Cantor and Schimmel, *Biophysical Chemistry Part I: The Conformation of Biological Macromolecules* (1980). "Primary structure" refers to the amino acid sequence of a particular peptide. "Secondary  
25 structure" refers to locally ordered, three dimensional structures within a polypeptide. These structures are commonly known as domains. Domains are portions of a polypeptide that form a compact unit of the polypeptide and are typically 25 to approximately 500 amino acids long. Typical domains are made up of sections of lesser organization such as stretches of  $\beta$ -sheet and  $\alpha$ -helices. "Tertiary structure" refers to the complete three dimensional  
30 structure of a polypeptide monomer. "Quaternary structure" refers to the three dimensional structure formed by the noncovalent association of independent tertiary units. Anisotropic terms are also known as energy terms.

[69] A "label" or a "detectable moiety" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include  $^{32}\text{P}$ , fluorescent dyes, electron-dense reagents, enzymes (*e.g.*, as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins for which ant  
5 or 7 can be made detectable, *e.g.*, by incorporating a radiolabel into the peptide, and used to detect antibodies specifically reactive with the peptide).

[70] A "labeled nucleic acid probe or oligonucleotide" is one that is bound, either covalently, through a linker or a chemical bond, or noncovalently, through ionic, van der Waals, electrostatic, or hydrogen bonds to a label such that the presence of the probe may be  
10 detected by detecting the presence of the label bound to the probe.

[71] As used herein a "nucleic acid probe or oligonucleotide" is defined as a nucleic acid capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (*i.e.*, A, G, C,  
15 or T) or modified bases (7-deazaguanosine, inosine, etc.). In addition, the bases in a probe may be joined by a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. Thus, for example, probes may be peptide nucleic acids in which the constituent bases are joined by peptide bonds rather than phosphodiester linkages. It will be understood by one of skill in the art that probes may bind target sequences lacking complete  
20 complementarity with the probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labeled as with isotopes, chromophores, lumiphores, chromogens, or indirectly labeled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the select sequence or subsequence.

[72] The term "recombinant" when used with reference, *e.g.*, to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-  
30 recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

[73] The term "heterologous" when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically

recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, *e.g.*, a promoter from one source and a coding region from another source. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature  
5 (*e.g.*, a fusion protein).

[74] A "promoter" is defined as an array of nucleic acid control sequences that direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor  
10 elements, which can be located as much as several thousand base pairs from the start site of transcription. A "constitutive" promoter is a promoter that is active under most environmental and developmental conditions. An "inducible" promoter is a promoter that is active under environmental or developmental regulation. The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as a  
15 promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

[75] An "expression vector" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a  
20 particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

[76] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that  
25 are the same or have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, about 70% identity, preferably 75%, 80%, 85%, 90%, or 95% identity over a specified region, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual  
30 alignment and visual inspection. Such sequences are then said to be "substantially identical." This definition also refers to the complement of a test sequence. Preferably, the identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 50-100 amino acids or nucleotides in length.

[77] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[78] A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

[79] A preferred example of algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nuc. Acids Res.* 25:3389-3402 (1997) and Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as



far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[80] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[81] An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequence.

[82] The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under

stringent hybridization conditions when that sequence is present in a complex mixture (*e.g.*, total cellular or library DNA or RNA).

[83] The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acid, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10° C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength pH. The  $T_m$  is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at  $T_m$ , 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30° C for short probes (*e.g.*, 10 to 50 nucleotides) and at least about 60° C for long probes (*e.g.*, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, optionally 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C. Such washes can be performed for 5, 15, 30, 60, 120, or more minutes. For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C - 95°C for 30 sec - 2 min., an annealing phase lasting 30 sec. - 2 min., and an extension phase of about 72°C for 1 - 2 min.

[84] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon

degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice  
5 background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

[85] "Antibody" refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta,  
10 epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

[86] An exemplary immunoglobulin (antibody) structural unit comprises a  
15 tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain ( $V_L$ ) and variable heavy chain ( $V_H$ ) refer to these light and heavy chains respectively.

[87] Antibodies exist, *e.g.*, as intact immunoglobulins or as a number of well-  
20 characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce  $F(ab)'_2$ , a dimer of Fab which itself is a light chain joined to  $V_H-C_H1$  by a disulfide bond. The  $F(ab)'_2$  may be reduced under mild conditions to break the disulfide linkage in the hinge region,  
25 thereby converting the  $F(ab)'_2$  dimer into an Fab' monomer. The Fab' monomer is essentially Fab with part of the hinge region (*see Fundamental Immunology* (Paul ed., 3d ed. 1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used  
30 herein, also includes antibody fragments either produced by the modification of whole antibodies, or those synthesized *de novo* using recombinant DNA methodologies (*e.g.*, single chain Fv) or those identified using phage display libraries (*see, e.g.*, McCafferty *et al.*, *Nature* 348:552-554 (1990)).

[88] For preparation of monoclonal or polyclonal antibodies, any technique known in the art can be used (*see, e.g.*, Kohler & Milstein, *Nature* 256:495-497 (1975); Kozbor *et al.*, *Immunology Today* 4: 72 (1983); Cole *et al.*, pp. 77-96 in *Monoclonal Antibodies and Cancer Therapy* (1985)). Techniques for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized antibodies. Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (*see, e.g.*, McCafferty *et al.*, *Nature* 348:552-554 (1990); Marks *et al.*, *Biotechnology* 10:779-783 (1992)).

[89] A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, *e.g.*, an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

[90] An "anti-GPCR" antibody is an antibody or antibody fragment that specifically binds a polypeptide encoded by a GPCR gene, cDNA, or a subsequence thereof.

[91] The term "immunoassay" is an assay that uses an antibody to specifically bind an antigen. The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the antigen.

[92] The phrase "specifically (or selectively) binds" to an antibody or "specifically (or selectively) immunoreactive with," when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and do not substantially bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies raised to a particular GPCR can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with the GPCR, and not with other proteins, except for polymorphic variants, orthologs, and alleles of the GPCR. This selection may be achieved by subtracting out antibodies that cross-react with GPCR molecules. A variety of immunoassay formats

may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (*see, e.g., Harlow & Lane, Antibodies, A Laboratory Manual* (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity). Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background. Antibodies that react only with a particular GPCR ortholog, *e.g.,* from specific species such as rat, mouse, or human, can also be made as described above, by subtracting out antibodies that bind to the same GPCR from another species.

10        [93]    The phrase "selectively associates with" refers to the ability of a nucleic acid to "selectively hybridize" with another as defined above, or the ability of an antibody to "selectively (or specifically) bind to a protein, as defined above.

#### Isolation of nucleic acids encoding GPCRs

##### 15            A. General recombinant DNA methods

      [94]    This invention relies on routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook *et al., Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al., eds., 1994*)).

      [95]    For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp). These are estimates derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons (kDa) or amino acid residue numbers. Proteins sizes are estimated from gel electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

      [96]    Oligonucleotides that are not commercially available can be chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Letts.* 22:1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et. al., Nucleic Acids Res.* 12:6159-6168 (1984). Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* 255:137-149 (1983).

[97] The sequence of the cloned genes and synthetic oligonucleotides can be verified after cloning using, *e.g.*, the chain termination method for sequencing double-stranded templates of Wallace *et al.*, *Gene* 16:21-26 (1981).

5                    *B. Cloning methods for the isolation of nucleotide sequences encoding GPCRs*

[98] In general, the nucleic acid sequences encoding GPCRs and related nucleic acid sequence homologs are cloned from cDNA and genomic DNA libraries by hybridization with a probe, or isolated using amplification techniques with oligonucleotide primers. For example, GPCR sequences are typically isolated from mammalian nucleic acid (genomic or  
10 cDNA) libraries by hybridizing with a nucleic acid probe, the sequence of which can be derived from SEQ ID NOS:1, 3, 5, 7, 9, or 11. Suitable tissues from which GPCR RNA and cDNA can be isolated include, *e.g.*, bone marrow, hypothalamus, spleen, colon, adipose, basal ganglia, skin, and other tissues.

[99] Amplification techniques using primers can also be used to amplify and isolate  
15 GPCR nucleic acids from DNA or RNA. Examples of suitable primers for amplification of specific GPCRs include those set forth in the Example Section (*see, e.g.*, Dieffenbach & Dveksler, *PCR Primer: A Laboratory Manual* (1995)). These primers can be used, *e.g.*, to amplify either the full length sequence or a probe of one to several hundred nucleotides, which is then used to screen a mammalian library for full-length GPCRs.

20            [100] Nucleic acids encoding GPCRs can also be isolated from expression libraries using antibodies as probes. Such polyclonal or monoclonal antibodies can be raised using the sequence of SEQ ID NOs:2, 4, 6, 8, 10, or 12.

[101] GPCR polymorphic variants, alleles, and interspecies homologs that are substantially identical to a GPCR can be isolated using GPCR nucleic acid probes, and  
25 oligonucleotides under stringent hybridization conditions, by screening libraries. Alternatively, expression libraries can be used to clone GPCRs and GPCR polymorphic variants, alleles, and interspecies homologs, by detecting expressed homologs immunologically with antisera or purified antibodies made against GPCRs, which also recognize and selectively bind to the GPCR homolog.

30            [102] To make a cDNA library, one should choose a source that is rich in GPCR mRNA. The mRNA is then made into cDNA using reverse transcriptase, ligated into a recombinant vector, and transfected into a recombinant host for propagation, screening and cloning. Methods for making and screening cDNA libraries are well known (*see, e.g.*, Gubler & Hoffman, *Gene* 25:263-269 (1983); Sambrook *et al., supra*; Ausubel *et al., supra*).

[103] For a genomic library, the DNA is extracted from the tissue and either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb. The fragments are then separated by gradient centrifugation from undesired sizes and are constructed in bacteriophage lambda vectors. These vectors and phage are packaged *in vitro*.  
5 Recombinant phage are analyzed by plaque hybridization as described in Benton & Davis, *Science* 196:180-182 (1977). Colony hybridization is carried out as generally described in Grunstein *et al.*, *Proc. Natl. Acad. Sci. USA.*, 72:3961-3965 (1975).

[104] An alternative method of isolating GPCR nucleic acids and their homologs combines the use of synthetic oligonucleotide primers and amplification of an RNA or DNA  
10 template (*see* U.S. Patents 4,683,195 and 4,683,202; *PCR Protocols: A Guide to Methods and Applications* (Innis *et al.*, eds, 1990)). Methods such as polymerase chain reaction (PCR) and ligase chain reaction (LCR) can be used to amplify nucleic acid sequences of GPCRs directly from mRNA, from cDNA, from genomic libraries or cDNA libraries. Degenerate oligonucleotides can be designed to amplify GPCR homologs using the sequences provided  
15 herein. Restriction endonuclease sites can be incorporated into the primers. Polymerase chain reaction or other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of GPCR-encoding mRNA in physiological samples, for nucleic acid sequencing, or for other purposes. Genes amplified by the PCR reaction can  
20 be purified from agarose gels and cloned into an appropriate vector.

[105] Gene expression of GPCRs can also be analyzed by techniques known in the art, *e.g.*, reverse transcription and amplification of mRNA, isolation of total RNA or poly A<sup>+</sup> RNA, northern blotting, dot blotting, *in situ* hybridization, RNase protection, probing DNA microchip arrays, and the like. In one embodiment, high density oligonucleotide analysis  
25 technology (*e.g.*, GeneChip™) is used to identify homologs and polymorphic variants of the GPCRs of the invention. In the case where the homologs being identified are linked to a known disease, they can be used with GeneChip™ as a diagnostic tool in detecting the disease in a biological sample, *see, e.g.*, Gunthand *et al.*, *AIDS Res. Hum. Retroviruses* 14: 869-876 (1998); Kozal *et al.*, *Nat. Med.* 2:753-759 (1996); Matson *et al.*, *Anal. Biochem.* 224:110-106 (1995); Lockhart *et al.*, *Nat. Biotechnol.* 14:1675-1680 (1996); Gingeras *et al.*, *Genome Res.* 8:435-448 (1998); Hacia *et al.*, *Nucleic Acids Res.* 26:3865-3866 (1998).  
30

[106] Synthetic oligonucleotides can be used to construct recombinant GPCR genes for use as probes or for expression of protein. This method is performed using a series of

overlapping oligonucleotides usually 40-120 bp in length, representing both the sense and nonsense strands of the gene. These DNA fragments are then annealed, ligated and cloned. Alternatively, amplification techniques can be used with precise primers to amplify a specific subsequence of the GPCR nucleic acid. The specific subsequence is then ligated into an expression vector.

[107] The nucleic acid encoding a GPCR is typically cloned into intermediate vectors before transformation into prokaryotic or eukaryotic cells for replication and/or expression. These intermediate vectors are typically prokaryote vectors, *e.g.*, plasmids, or shuttle vectors.

[108] Optionally, nucleic acids encoding chimeric proteins comprising GPCRs or domains thereof can be made according to standard techniques. For example, a domain such as ligand binding domain, an extracellular domain, a transmembrane domain (*e.g.*, one comprising seven transmembrane regions and corresponding extracellular and cytosolic loops), the transmembrane domain and a cytoplasmic domain, an active site, a subunit association region, etc., can be covalently linked to a heterologous protein. For example, an extracellular domain can be linked to a heterologous GPCR transmembrane domain, or a heterologous GPCR extracellular domain can be linked to a transmembrane domain. Other heterologous proteins of choice include, *e.g.*, green fluorescent protein, luciferase, or  $\beta$ -gal.

#### C. Expression in prokaryotes and eukaryotes

[109] To obtain high level expression of a cloned gene or nucleic acid, such as those cDNAs encoding GPCRs, one typically subclones a GPCR into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and if for a nucleic acid encoding a protein, a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, *e.g.*, in Sambrook *et al.* and Ausubel *et al.* Bacterial expression systems for expressing the GPCR protein are available in, *e.g.*, *E. coli*, *Bacillus sp.*, and *Salmonella* (Palva *et al.*, *Gene* 22:229-235 (1983); Mosbach *et al.*, *Nature* 302:543-545 (1983). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available. In one embodiment, the eukaryotic expression vector is an adenoviral vector, an adeno-associated vector, or a retroviral vector.



[110] The promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is optionally positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

[111] In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of the GPCR encoding nucleic acid in host cells. A typical expression cassette thus contains a promoter operably linked to the nucleic acid sequence encoding a GPCR and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. The nucleic acid sequence encoding a GPCR may typically be linked to a cleavable signal peptide sequence to promote secretion of the encoded protein by the transformed cell. Such signal peptides would include, among others, the signal peptides from tissue plasminogen activator, insulin, and neuron growth factor, and juvenile hormone esterase of *Heliothis virescens*. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

[112] In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

[113] The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems such as GST and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, e.g., c-myc.

[114] Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, e.g., SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A<sup>+</sup>, pMTO10/A<sup>+</sup>, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma

virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[115] Some expression systems have markers that provide gene amplification such as thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase.

5 Alternatively, high yield expression systems not involving gene amplification are also suitable, such as using a baculovirus vector in insect cells, with a GPCR-encoding sequence under the direction of the polyhedrin promoter or other strong baculovirus promoters.

[116] The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of  
10 bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are optionally chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary.

15 [117] Standard transfection methods are used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of GPCR protein, which are then purified using standard techniques (*see, e.g., Colley et al., J. Biol. Chem.* 264:17619-17622 (1989); *Guide to Protein Purification*, in *Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to  
20 standard techniques (*see, e.g., Morrison, J. Bact.* 132:349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101:347-362 (Wu *et al.*, eds, 1983).

[118] Any of the well known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, plasma  
25 vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (*see, e.g., Sambrook et al., supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing a GPCR.

30 [119] After the expression vector is introduced into the cells, the transfected cells are cultured under conditions favoring expression of a GPCR, which is recovered from the culture using standard techniques identified below.

### Purification of GPCRs

[120] Either naturally occurring or recombinant GPCRs can be purified for use in functional assays. Optionally, recombinant GPCRs are purified. Naturally occurring GPCRs are purified, *e.g.*, from any suitable tissue or cell expressing naturally occurring GPCRs.

- 5 Recombinant GPCRs are purified from any suitable bacterial or eukaryotic expression system, *e.g.*, CHO cells or insect cells.

[121] A GPCR may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others (*see, e.g.*, Scopes, *Protein*  
10 *Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al.*, *supra*; and Sambrook *et al.*, *supra*).

[122] A number of procedures can be employed when a recombinant GPCR is being purified. For example, proteins having established molecular adhesion properties can be reversible fused to a GPCR. With the appropriate ligand, a GPCR can be selectively  
15 adsorbed to a purification column and then freed from the column in a relatively pure form. The fused protein is then removed by enzymatic activity. Finally, a GPCR could be purified using immunoaffinity columns.

#### *A. Purification of GPCRs from recombinant cells*

20 [123] Recombinant proteins are expressed by transformed bacteria or eukaryotic cells such as CHO cells or insect cells in large amounts, typically after promoter induction; but expression can be constitutive. Promoter induction with IPTG is a one example of an inducible promoter system. Cells are grown according to standard procedures in the art. Fresh or frozen cells are used for isolation of protein.

25 [124] Proteins expressed in bacteria may form insoluble aggregates ("inclusion bodies"). Several protocols are suitable for purification of GPCR inclusion bodies. For example, purification of inclusion bodies typically involves the extraction, separation and/or purification of inclusion bodies by disruption of bacterial cells, *e.g.*, by incubation in a buffer of 50 mM TRIS/HCL pH 7.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mM ATP, and 1  
30 mM PMSF. The cell suspension can be lysed using 2-3 passages through a French Press, homogenized using a Polytron (Brinkman Instruments) or sonicated on ice. Alternate methods of lysing bacteria are apparent to those of skill in the art (*see, e.g.*, Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*).

[125] If necessary, the inclusion bodies are solubilized, and the lysed cell suspension is typically centrifuged to remove unwanted insoluble matter. Proteins that formed the inclusion bodies may be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to urea (from about 4 M to about 8 M), formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Some solvents which are capable of solubilizing aggregate-forming proteins, for example SDS (sodium dodecyl sulfate), 70% formic acid, are inappropriate for use in this procedure due to the possibility of irreversible denaturation of the proteins, accompanied by a lack of immunogenicity and/or activity. Although guanidine hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant, allowing re-formation of immunologically and/or biologically active protein. Other suitable buffers are known to those skilled in the art. The GPCR is separated from other bacterial proteins by standard separation techniques, *e.g.*, with Ni-NTA agarose resin.

[126] Alternatively, it is possible to purify the GPCR from bacteria periplasm. After lysis of the bacteria, when the GPCR is exported into the periplasm of the bacteria, the periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to skill in the art. To isolate recombinant proteins from the periplasm, the bacterial cells are centrifuged to form a pellet. The pellet is resuspended in a buffer containing 20% sucrose. To lyse the cells, the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM MgSO<sub>4</sub> and kept in an ice bath for approximately 10 minutes. The cell suspension is centrifuged and the supernatant decanted and saved. The recombinant proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

25

### *B. Standard protein separation techniques for purifying GPCRs*

#### Solubility fractionation

[127] Often as an initial step, particularly if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol includes adding saturated ammonium

sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This concentration will precipitate the most hydrophobic of proteins. The precipitate is then discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, either through dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

#### 10                                    Size differential filtration

[128] The molecular weight of a GPCR can be used to isolated it from proteins of greater and lesser size using ultrafiltration through membranes of different pore size (for example, Amicon or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of the protein of interest. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

#### 20                                    Column chromatography

[129] GPCRs can also be separated from other proteins on the basis of its size, net surface charge, hydrophobicity, and affinity for ligands. In addition, antibodies raised against proteins can be conjugated to column matrices and the proteins immunopurified. All of these methods are well known in the art. It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (e.g., Pharmacia Biotech).

#### **Immunological detection of GPCRs**

[130] In addition to the detection of GPCR genes and gene expression using nucleic acid hybridization technology, one can also use immunoassays to detect GPCRs, e.g., to identify cells such as kidney cells, liver cells, adipocytes, hypothalamus cells, spleen cells, or colon cells, and variants of GPCRs. Immunoassays can be used to qualitatively or quantitatively analyze GPCRs. A general overview of the applicable technology can be found in Harlow & Lane, *Antibodies: A Laboratory Manual* (1988).

### A. Antibodies to GPCRs

[131] Methods of producing polyclonal and monoclonal antibodies that react specifically with GPCRs are known to those of skill in the art (*see, e.g., Coligan, Current Protocols in Immunology* (1991); Harlow & Lane, *supra*; Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986); and Kohler & Milstein, *Nature* 256:495-497 (1975)). Such techniques include antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors, as well as preparation of polyclonal and monoclonal antibodies by immunizing rabbits or mice (*see, e.g., Huse et al., Science* 246:1275-1281 (1989); Ward *et al., Nature* 341:544-546 (1989)). Such antibodies can be used for therapeutic and diagnostic applications, *e.g., in the treatment and/or detection of any of the GPCR-associated diseases or conditions described herein.*

[132] A number of GPCRs comprising immunogens may be used to produce antibodies specifically reactive with GPCRs. For example, a recombinant GPCR or an antigenic fragment thereof, is isolated as described herein. Recombinant protein can be expressed in eukaryotic or prokaryotic cells as described above, and purified as generally described above. Recombinant protein is the preferred immunogen for the production of monoclonal or polyclonal antibodies. Alternatively, a synthetic peptide derived from the sequences disclosed herein and conjugated to a carrier protein can be used as an immunogen. Naturally occurring protein may also be used either in pure or impure form. The product is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies may be generated, for subsequent use in immunoassays to measure the protein.

[133] Methods of production of polyclonal antibodies are known to those of skill in the art. An inbred strain of mice (*e.g., BALB/C mice*) or rabbits is immunized with the protein using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the GPCR. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired (*see Harlow & Lane, supra*).

[134] Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (*see Kohler & Milstein,*

*Eur. J. Immunol.* 6:511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the  
5 monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined by Huse *et al.*, *Science* 246:1275-1281 (1989).

- 10 [135] Monoclonal antibodies and polyclonal sera are collected and titered against the immunogen protein in an immunoassay, for example, a solid phase immunoassay with the immunogen immobilized on a solid support. Typically, polyclonal antisera with a titer of  $10^4$  or greater are selected and tested for their cross reactivity against non-GPCR proteins or even other related proteins from other organisms, using a competitive binding immunoassay.
- 15 Specific polyclonal antisera and monoclonal antibodies will usually bind with a  $K_d$  of at least about 0.1 mM, more usually at least about 1  $\mu$ M, optionally at least about 0.1  $\mu$ M or better, and optionally 0.01  $\mu$ M or better.

- [136] Once GPCR specific antibodies are available, GPCRs can be detected by a variety of immunoassay methods. For a review of immunological and immunoassay  
20 procedures, see *Basic and Clinical Immunology* (Stites & Terr eds., 7th ed. 1991). Moreover, the immunoassays of the present invention can be performed in any of several configurations, which are reviewed extensively in *Enzyme Immunoassay* (Maggio, ed., 1980); and Harlow & Lane, *supra*.

25 *B. Immunological binding assays*

- [137] GPCRs can be detected and/or quantified using any of a number of well recognized immunological binding assays (*see, e.g.*, U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also *Methods in Cell Biology: Antibodies in Cell Biology*, volume 37 (Asai, ed. 1993); *Basic and Clinical*  
30 *Immunology* (Stites & Terr, eds., 7th ed. 1991). Immunological binding assays (or immunoassays) typically use an antibody that specifically binds to a protein or antigen of choice (in this case the GPCR or antigenic subsequence thereof). The antibody (*e.g.*, anti-GPCR) may be produced by any of a number of means well known to those of skill in the art and as described above.

[138] Immunoassays also often use a labeling agent to specifically bind to and label the complex formed by the antibody and antigen. The labeling agent may itself be one of the moieties comprising the antibody/antigen complex. Thus, the labeling agent may be a labeled GPCR polypeptide or a labeled anti-GPCR antibody. Alternatively, the labeling agent may be a third moiety, such as a secondary antibody, that specifically binds to the antibody/ GPCR complex (a secondary antibody is typically specific to antibodies of the species from which the first antibody is derived). Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the label agent. These proteins exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species (*see, e.g., Kronval et al., J. Immunol.* 111:1401-1406 (1973); *Akerstrom et al., J. Immunol.* 135:2589-2542 (1985)). The labeling agent can be modified with a detectable moiety, such as biotin, to which another molecule can specifically bind, such as streptavidin. A variety of detectable moieties are well known to those skilled in the art.

[139] Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, optionally from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, antigen, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

#### Non-competitive assay formats

[140] Immunoassays for detecting GPCRs in samples may be either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of antigen is directly measured. In one preferred "sandwich" assay, for example, the anti-GPCR antibodies can be bound directly to a solid substrate on which they are immobilized. These immobilized antibodies then capture GPCRs present in the test sample. The GPCR is thus immobilized is then bound by a labeling agent, such as a second GPCR antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second or third antibody is typically modified with a detectable moiety, such as biotin, to which another molecule specifically binds, *e.g., streptavidin*, to provide a detectable moiety.



### Competitive assay formats

[141] In competitive assays, the amount of GPCR present in the sample is measured indirectly by measuring the amount of a known, added (exogenous) GPCR displaced (competed away) from an anti-GPCR antibody by the unknown GPCR present in a sample. In one competitive assay, a known amount of GPCR is added to a sample and the sample is then contacted with an antibody that specifically binds to the GPCR. The amount of exogenous GPCR bound to the antibody is inversely proportional to the concentration of GPCR present in the sample. In a particularly preferred embodiment, the antibody is immobilized on a solid substrate. The amount of GPCR bound to the antibody may be determined either by measuring the amount of GPCR present in a GPCR/antibody complex, or alternatively by measuring the amount of remaining uncomplexed protein. The amount of GPCR may be detected by providing a labeled GPCR molecule.

[142] A hapten inhibition assay is another preferred competitive assay. In this assay the known GPCR, is immobilized on a solid substrate. A known amount of anti-GPCR antibody is added to the sample, and the sample is then contacted with the immobilized GPCR. The amount of anti-GPCR antibody bound to the known immobilized GPCR is inversely proportional to the amount of GPCR present in the sample. Again, the amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. Detection may be direct where the antibody is labeled or indirect by the subsequent addition of a labeled moiety that specifically binds to the antibody as described above.

### Cross-reactivity determinations

[143] Immunoassays in the competitive binding format can also be used for crossreactivity determinations. For example, a protein at least partially encoded by SEQ ID NOs:1, 3, 5, 7, 9, or 11, can be immobilized to a solid support. Proteins (*e.g.*, GPCR proteins and homologs) are added to the assay that compete for binding of the antisera to the immobilized antigen. The ability of the added proteins to compete for binding of the antisera to the immobilized protein is compared to the ability of GPCRs encoded by SEQ ID NO:1, 3, 5, 7, 9, or 11 to compete with itself. The percent crossreactivity for the above proteins is calculated, using standard calculations. Those antisera with less than 10% crossreactivity with each of the added proteins listed above are selected and pooled. The cross-reacting

antibodies are optionally removed from the pooled antisera by immunoabsorption with the added considered proteins, *e.g.*, distantly related homologs.

[144] The immunoabsorbed and pooled antisera are then used in a competitive binding immunoassay as described above to compare a second protein, thought to be perhaps an allele or polymorphic variant of a GPCR, to the immunogen protein (*i.e.*, the GPCR of SEQ ID NOS:2, 4, 6, 8, 10, or 12). In order to make this comparison, the two proteins are each assayed at a wide range of concentrations and the amount of each protein required to inhibit 50% of the binding of the antisera to the immobilized protein is determined. If the amount of the second protein required to inhibit 50% of binding is less than 10 times the amount of the protein encoded by SEQ ID NOS:1, 3, 5, 7, 9, or 11 that is required to inhibit 50% of binding, then the second protein is said to specifically bind to the polyclonal antibodies generated to a GPCR immunogen.

#### Other assay formats

[145] Western blot (immunoblot) analysis is used to detect and quantify the presence of GPCR in the sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind GPCR. The anti-GPCR antibodies specifically bind to the GPCR on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (*e.g.*, labeled sheep anti-mouse antibodies) that specifically bind to the anti-GPCR antibodies.

[146] Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (*see Monroe et al., Amer. Clin. Prod. Rev. 5:34-41 (1986)*).

#### Reduction of non-specific binding

[147] One of skill in the art will appreciate that it is often desirable to minimize non-specific binding in immunoassays. Particularly, where the assay involves an antigen or antibody immobilized on a solid substrate it is desirable to minimize the amount of non-specific binding to the substrate. Means of reducing such non-specific binding are well known to those of skill in the art. Typically, this technique involves coating the substrate with a proteinaceous composition. In particular, protein compositions such as bovine serum

albumin (BSA), nonfat powdered milk, and gelatin are widely used with powdered milk being most preferred.

### Labels

5           [148] The particular label or detectable group used in the assay is not a critical aspect of the invention, as long as it does not significantly interfere with the specific binding of the antibody used in the assay. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays and, in general, most any label useful in such methods can be  
10           applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.*, DYNABEADS<sup>TM</sup>), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (*e.g.*, <sup>3</sup>H, <sup>125</sup>I, <sup>35</sup>S, <sup>14</sup>C, or <sup>32</sup>P), enzymes (*e.g.*, horse radish  
15           peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads (*e.g.*, polystyrene, polypropylene, latex, etc.).

          [149] The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of  
20           labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

          [150] Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to the molecule. The ligand then binds to  
25           another molecules (*e.g.*, streptavidin) molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. The ligands and their targets can be used in any suitable combination with antibodies that recognize GPCRs, or secondary antibodies that recognize anti-GPCR.

30           [151] The molecules can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidotases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent

compounds include luciferin, and 2,3-dihydrophthalazinediones, *e.g.*, luminol. For a review of various labeling or signal producing systems that may be used, see U.S. Patent No. 4,391,904.

[152] Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

[153] Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen-coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

## Assays for modulators of GPCRs

### *A. Assays for GPCR activity*

[154] GPCRs and their alleles and polymorphic variants are G-protein coupled receptors that participate in signal transduction and are associated with cellular function (*e.g.*, detection of ligands) in a variety of cells, *e.g.*, kidney, liver, colon, adipose, hypothalamus, and other cells. The activity of GPCR polypeptides can be assessed using a variety of *in vitro* and *in vivo* assays to determine functional, chemical, and physical effects, *e.g.*, measuring ligand binding (*e.g.*, radioactive ligand binding), second messengers (*e.g.*, cAMP, cGMP, IP<sub>3</sub>, DAG, or Ca<sup>2+</sup>), ion flux, phosphorylation levels, transcription levels, neurotransmitter levels, and the like. Furthermore, such assays can be used to test for inhibitors and activators of a GPCR. Modulators can also be genetically altered versions of a GPCR. Screening assays of the invention are used to identify modulators that can be used as therapeutic co, *e.g.*, antibodies to GPCRs and antagonists of GPCR activity.

[155] The GPCR of the assay will be selected from a polypeptide having a sequence of SEQ ID NOs:2, 4, 6, 8, 10 or 12, or conservatively modified variant thereof.

Alternatively, the GPCR of the assay will be derived from a eukaryote and include an amino acid subsequence having amino acid sequence identity to SEQ ID NOs:2, 4, 6, 8, 10, or 12.

5 Generally, the amino acid sequence identity will be at least 70%, optionally at least 80%, optionally at least 90-95%. Optionally, the polypeptide of the assays will comprise a domain of a GPCR, such as an extracellular domain, transmembrane domain, cytoplasmic domain, ligand binding domain, subunit association domain, active site, and the like. Either a GPCR or a domain thereof can be covalently linked to a heterologous protein to create a chimeric  
10 protein used in the assays described herein.

[156] Modulators of GPCR activity are tested using GPCR polypeptides as described above, either recombinant or naturally occurring. The protein can be isolated, expressed in a cell, expressed in a membrane derived from a cell, expressed in tissue or in an animal, either recombinant or naturally occurring. For example, neurons, colon cells, spleen  
15 cells, adipocytes, skin cells, transformed cells, or membranes can be used. Modulation is tested using one of the *in vitro* or *in vivo* assays described herein. Signal transduction can also be examined *in vitro* with soluble or solid state reactions, using a chimeric molecule such as an extracellular domain of a receptor covalently linked to a heterologous signal transduction domain, or a heterologous extracellular domain covalently linked to the  
20 transmembrane and or cytoplasmic domain of a receptor. Gene amplification can also be examined. Furthermore, ligand-binding domains of the protein of interest can be used *in vitro* in soluble or solid state reactions to assay for ligand binding.

[157] Ligand binding to GPCR, a domain, or chimeric protein can be tested in solution, in a bilayer membrane, attached to a solid phase, in a lipid monolayer, or in vesicles.  
25 Binding of a modulator can be tested using, *e.g.*, changes in spectroscopic characteristics (*e.g.*, fluorescence, absorbance, refractive index) hydrodynamic (*e.g.*, shape), chromatographic, or solubility properties.

[158] Receptor-G-protein interactions can also be examined. For example, binding of the G-protein to the receptor or its release from the receptor can be examined. For  
30 example, in the absence of GTP, an activator will lead to the formation of a tight complex of a G protein (all three subunits) with the receptor. This complex can be detected in a variety of ways, as noted above. Such an assay can be modified to search for inhibitors. Add an activator to the receptor and G protein in the absence of GTP, form a tight complex, and then screen for inhibitors by looking at dissociation of the receptor-G protein complex. In the

presence of GTP, release of the alpha subunit of the G protein from the other two G protein subunits serves as a criterion of activation.

5 [159] An activated or inhibited G-protein will in turn alter the properties of downstream effectors such as proteins, enzymes, and channels. The classic examples are the activation of cGMP phosphodiesterase by transducin in the visual system, adenylate cyclase by the stimulatory G-protein, phospholipase C by Gq and other cognate G proteins, and modulation of diverse channels by Gi and other G proteins. Downstream consequences can also be examined such as generation of diacyl glycerol and IP<sub>3</sub> by phospholipase C, and in turn, for calcium mobilization by IP<sub>3</sub>.

10 [160] Activated GPCR receptors become substrates for kinases that phosphorylate the C-terminal tail of the receptor (and possibly other sites as well). Thus, activators will promote the transfer of <sup>32</sup>P from gamma-labeled GTP to the receptor, which can be assayed with a scintillation counter. The phosphorylation of the C-terminal tail will promote the binding of arrestin-like proteins and will interfere with the binding of G-proteins. The  
15 kinase/arrestin pathway plays a key role in the desensitization of many GPCR receptors. For a general review of GPCR signal transduction and methods of assaying signal transduction, see, e.g., *Methods in Enzymology*, vols. 237 and 238 (1994) and volume 96 (1983); Bourne *et al.*, *Nature* 10:349:117-27 (1991); Bourne *et al.*, *Nature* 348:125-32 (1990); Pitcher *et al.*, *Annu. Rev. Biochem.* 67:653-92 (1998).

20 [161] Samples or assays that are treated with a potential GPCR inhibitor or activator are compared to control samples without the test compound, to examine the extent of modulation. Control samples (untreated with activators or inhibitors) are assigned a relative GPCR activity value of 100. Inhibition of a GPCR is achieved when the GPCR activity value relative to the control is about 90%, optionally 50%, optionally 25-0%. Activation of a  
25 GPCR is achieved when the GPCR activity value relative to the control is 110%, optionally 150%, 200-500%, or 1000-2000%.

[162] Changes in ion flux may be assessed by determining changes in polarization (*i.e.*, electrical potential) of the cell or membrane expressing a GPCR. One means to determine changes in cellular polarization is by measuring changes in current (thereby  
30 measuring changes in polarization) with voltage-clamp and patch-clamp techniques, *e.g.*, the "cell-attached" mode, the "inside-out" mode, and the "whole cell" mode (see, *e.g.*, Ackerman *et al.*, *New Engl. J. Med.* 336:1575-1595 (1997)). Whole cell currents are conveniently determined using the standard methodology (see, *e.g.*, Hamil *et al.*, *Pflugers. Archiv.* 391:85 (1981). Other known assays include: radiolabeled ion flux assays and fluorescence assays

using voltage-sensitive dyes (*see, e.g., Vestergaard-Bogind et al., J. Membrane Biol.* 88:67-75 (1988); Gonzales & Tsien, *Chem. Biol.* 4:269-277 (1997); Daniel *et al., J. Pharmacol. Meth.* 25:185-193 (1991); Holevinsky *et al., J. Membrane Biology* 137:59-70 (1994)). Generally, the compounds to be tested are present in the range from 1 pM to 100 mM.

5           [163] The effects of the test compounds upon the function of the polypeptides can be measured by examining any of the parameters described above. Any suitable physiological change that affects GPCR activity can be used to assess the influence of a test compound on the polypeptides of this invention. When the functional consequences are determined using intact cells or animals, one can also measure a variety of effects such as transmitter release,  
10 hormone release, transcriptional changes to both known and uncharacterized genetic markers (*e.g.,* northern blots), changes in cell metabolism such as cell growth or pH changes, and changes in intracellular second messengers such as  $\text{Ca}^{2+}$ ,  $\text{IP}_3$  or cAMP.

          [164] Preferred assays for G-protein coupled receptors include cells that are loaded with ion or voltage sensitive dyes to report receptor activity. Assays for determining activity  
15 of such receptors can also use known agonists and antagonists for other G-protein coupled receptors as negative or positive controls to assess activity of tested compounds. In assays for identifying modulatory compounds (*e.g.,* agonists, antagonists), changes in the level of ions in the cytoplasm or membrane voltage will be monitored using an ion sensitive or membrane voltage fluorescent indicator, respectively. Among the ion-sensitive indicators  
20 and voltage probes that may be employed are those disclosed in the Molecular Probes 1997 Catalog. For G-protein coupled receptors, promiscuous G-proteins such as  $\text{G}\alpha_{15}$  and  $\text{G}\alpha_{16}$  can be used in the assay of choice (Wilkie *et al., Proc. Nat'l Acad. Sci. USA* 88:10049-10053 (1991)). Such promiscuous G-proteins allow coupling of a wide range of receptors to signal transduction pathways in heterologous cells.

25           [165] Receptor activation typically initiates subsequent intracellular events, *e.g.,* increases in second messengers such as  $\text{IP}_3$ , which releases intracellular stores of calcium ions. Activation of some G-protein coupled receptors stimulates the formation of inositol triphosphate ( $\text{IP}_3$ ) through phospholipase C-mediated hydrolysis of phosphatidylinositol (Berridge & Irvine, *Nature* 312:315-21 (1984)).  $\text{IP}_3$  in turn stimulates the release of  
30 intracellular calcium ion stores. Thus, a change in cytoplasmic calcium ion levels, or a change in second messenger levels such as  $\text{IP}_3$  can be used to assess G-protein coupled receptor function. Cells expressing such G-protein coupled receptors may exhibit increased cytoplasmic calcium levels as a result of contribution from both intracellular stores and via

activation of ion channels, in which case it may be desirable although not necessary to conduct such assays in calcium-free buffer, optionally supplemented with a chelating agent such as EGTA, to distinguish fluorescence response resulting from calcium release from internal stores.

5           [166] Other assays can involve determining the activity of receptors which, when activated, result in a change in the level of intracellular cyclic nucleotides, *e.g.*, cAMP or cGMP, by activating or inhibiting downstream effectors such as adenylate cyclase. There are cyclic nucleotide-gated ion channels, *e.g.*, rod photoreceptor cell channels and olfactory neuron channels that are permeable to cations upon activation by binding of cAMP or cGMP  
10 (*see, e.g., Altenhofen et al., Proc. Natl. Acad. Sci. U.S.A.* 88:9868-9872 (1991) and Dhallan *et al., Nature* 347:184-187 (1990)). In cases where activation of the receptor results in a decrease in cyclic nucleotide levels, it may be preferable to expose the cells to agents that increase intracellular cyclic nucleotide levels, *e.g.*, forskolin, prior to adding a receptor-activating compound to the cells in the assay. Cells for this type of assay can be made by co-  
15 transfection of a host cell with DNA encoding a cyclic nucleotide-gated ion channel, GPCR phosphatase and DNA encoding a receptor (*e.g.*, certain glutamate receptors, muscarinic acetylcholine receptors, dopamine receptors, serotonin receptors, and the like), which, when activated, causes a change in cyclic nucleotide levels in the cytoplasm.

          [167] In one embodiment, changes in intracellular cAMP or cGMP can be measured  
20 using immunoassays. The method described in Offermanns & Simon, *J. Biol. Chem.* 270:15175-15180 (1995) may be used to determine the level of cAMP. Also, the method described in Felley-Bosco *et al., Am. J. Resp. Cell and Mol. Biol.* 11:159-164 (1994) may be used to determine the level of cGMP. Further, an assay kit for measuring cAMP and/or cGMP is described in U.S. Patent 4,115,538, herein incorporated by reference.

25           [168] In another embodiment, phosphatidyl inositol (PI) hydrolysis can be analyzed according to U.S. Patent 5,436,128, herein incorporated by reference. Briefly, the assay involves labeling of cells with <sup>3</sup>H-myoinositol for 48 or more hrs. The labeled cells are treated with a test compound for one hour. The treated cells are lysed and extracted in chloroform-methanol-water after which the inositol phosphates were separated by ion  
30 exchange chromatography and quantified by scintillation counting. Fold stimulation is determined by calculating the ratio of cpm in the presence of agonist to cpm in the presence of buffer control. Likewise, fold inhibition is determined by calculating the ratio of cpm in the presence of antagonist to cpm in the presence of buffer control (which may or may not contain an agonist).



[169] In another embodiment, transcription levels can be measured to assess the effects of a test compound on signal transduction. A host cell containing the protein of interest is contacted with a test compound for a sufficient time to effect any interactions, and then the level of gene expression is measured. The amount of time to effect such interactions may be empirically determined, such as by running a time course and measuring the level of transcription as a function of time. The amount of transcription may be measured by using any method known to those of skill in the art to be suitable. For example, mRNA expression of the protein of interest may be detected using northern blots or their polypeptide products may be identified using immunoassays. Alternatively, transcription based assays using reporter gene may be used as described in U.S. Patent 5,436,128, herein incorporated by reference. The reporter genes can be, *e.g.*, chloramphenicol acetyltransferase, firefly luciferase, bacterial luciferase,  $\beta$ -galactosidase and alkaline phosphatase. Furthermore, the protein of interest can be used as an indirect reporter via attachment to a second reporter such as green fluorescent protein (*see, e.g.,* Mistili & Spector, *Nature Biotechnology* 15:961-964 (1997)).

[170] The amount of transcription is then compared to the amount of transcription in either the same cell in the absence of the test compound, or it may be compared with the amount of transcription in a substantially identical cell that lacks the protein of interest. A substantially identical cell may be derived from the same cells from which the recombinant cell was prepared but which had not been modified by introduction of heterologous DNA. Any difference in the amount of transcription indicates that the test compound has in some manner altered the activity of the protein of interest.

### *B. Modulators*

[171] The compounds tested as modulators of GPCRs can be any small chemical compound, or a biological entity, *e.g.*, a macromolecule such as a protein, sugar, nucleic acid or lipid. Alternatively, modulators can be genetically altered versions of a GPCR. Typically, test compounds will be small chemical molecules and peptides. Essentially any chemical compound can be used as a potential modulator or ligand in the assays of the invention, although most often compounds can be dissolved in aqueous or organic (especially DMSO-based) solutions are used. The assays are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (*e.g.*, in microtiter formats on microtiter plates in robotic

assays). It will be appreciated that there are many suppliers of chemical compounds, including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-Aldrich (St. Louis, MO), Fluka Chemika-Biochemica Analytika (Buchs Switzerland) and the like.

[172] In one preferred embodiment, high throughput screening methods involve providing a combinatorial chemical or peptide library containing a large number of potential therapeutic compounds (potential modulator or ligand compounds). Such "combinatorial chemical libraries" or "ligand libraries" are then screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

[173] A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

[174] Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (*see, e.g.*, U.S. Patent 5,010,175, Furka, *Int. J. Pept. Prot. Res.* 37:487-493 (1991) and Houghton *et al.*, *Nature* 354:84-88 (1991)). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (*e.g.*, PCT Publication No. WO 91/19735), encoded peptides (*e.g.*, PCT Publication WO 93/20242), random bio-oligomers (*e.g.*, PCT Publication No. WO 92/00091), benzodiazepines (*e.g.*, U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al.*, *Proc. Nat. Acad. Sci. USA* 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al.*, *J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann *et al.*, *J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen *et al.*, *J. Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho *et al.*, *Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell *et al.*, *J. Org. Chem.* 59:658 (1994)), nucleic acid libraries (*see* Ausubel, Berger and Sambrook, all *supra*), peptide nucleic acid libraries (*see, e.g.*, U.S. Patent 5,539,083), antibody libraries (*see, e.g.*, Vaughn *et al.*, *Nature Biotechnology*, 14(3):309-314 (1996) and PCT/US96/10287), carbohydrate libraries (*see,*

e.g., Liang *et al.*, *Science*, 274:1520-1522 (1996) and U.S. Patent 5,593,853), small organic molecule libraries (*see, e.g.*, benzodiazepines, Baum C&EN, Jan 18, page 33 (1993); isoprenoids, U.S. Patent 5,569,588; thiazolidinones and metathiazanones, U.S. Patent 5,549,974; pyrrolidines, U.S. Patents 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent 5,506,337; benzodiazepines, 5,288,514, and the like).

[175] Devices for the preparation of combinatorial libraries are commercially available (*see, e.g.*, 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA). In addition, numerous combinatorial libraries are themselves commercially available (*see, e.g.*, ComGenex, Princeton, N.J., Tripos, Inc., St. Louis, MO, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, etc.).

### *C. Solid State and soluble high throughput assays*

[176] In one embodiment the invention provide soluble assays using molecules such as a domain such as ligand binding domain, an extracellular domain, a transmembrane domain (*e.g.*, one comprising seven transmembrane regions and cytosolic loops), the transmembrane domain and a cytoplasmic domain, an active site, a subunit association region, etc.; a domain that is covalently linked to a heterologous protein to create a chimeric molecule; a GPCR; or a cell or tissue expressing a GPCR, either naturally occurring or recombinant. In another embodiment, the invention provides solid phase based *in vitro* assays in a high throughput format, where the domain, chimeric molecule, GPCR, or cell or tissue expressing a GPCR is attached to a solid phase substrate.

[177] In the high throughput assays of the invention, it is possible to screen up to several thousand different modulators or ligands in a single day. In particular, each well of a microtiter plate can be used to run a separate assay against a selected potential modulator, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter plate can assay about 100 (*e.g.*, 96) modulators. If 1536 well plates are used, then a single plate can easily assay from about 100- about 1500 different compounds. It is possible to assay several different plates per day; assay screens for up to about 6,000-20,000 different compounds is possible using the integrated systems of the invention.

[178] The molecule of interest can be bound to the solid state component, directly or indirectly, via covalent or non covalent linkage *e.g.*, via a tag. The tag can be any of a variety of components. In general, a molecule which binds the tag (a tag binder) is fixed to a solid

support, and the tagged molecule of interest (*e.g.*, the signal transduction molecule of interest) is attached to the solid support by interaction of the tag and the tag binder.

[179] A number of tags and tag binders can be used, based upon known molecular interactions well described in the literature. For example, where a tag has a natural binder, for example, biotin, protein A, or protein G, it can be used in conjunction with appropriate tag binders (avidin, streptavidin, neutravidin, the Fc region of an immunoglobulin, *etc.*) Antibodies to molecules with natural binders such as biotin are also widely available and appropriate tag binders; *see*, SIGMA Immunochemicals 1998 catalogue SIGMA, St. Louis MO).

[180] Similarly, any haptenic or antigenic compound can be used in combination with an appropriate antibody to form a tag/tag binder pair. Thousands of specific antibodies are commercially available and many additional antibodies are described in the literature. For example, in one common configuration, the tag is a first antibody and the tag binder is a second antibody which recognizes the first antibody. In addition to antibody-antigen interactions, receptor-ligand interactions are also appropriate as tag and tag-binder pairs. For example, agonists and antagonists of cell membrane receptors (*e.g.*, cell receptor-ligand interactions such as transferrin, c-kit, viral receptor ligands, cytokine receptors, chemokine receptors, interleukin receptors, immunoglobulin receptors and antibodies, the cadherein family, the integrin family, the selectin family, and the like; *see, e.g.*, Pigott & Power, *The Adhesion Molecule Facts Book I* (1993). Similarly, toxins and venoms, viral epitopes, hormones (*e.g.*, opiates, steroids, *etc.*), intracellular receptors (*e.g.* which mediate the effects of various small ligands, including steroids, thyroid hormone, retinoids and vitamin D; peptides), drugs, lectins, sugars, nucleic acids (both linear and cyclic polymer configurations), oligosaccharides, proteins, phospholipids and antibodies can all interact with various cell receptors.

[181] Synthetic polymers, such as polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, and polyacetates can also form an appropriate tag or tag binder. Many other tag/tag binder pairs are also useful in assay systems described herein, as would be apparent to one of skill upon review of this disclosure.

[182] Common linkers such as peptides, polyethers, and the like can also serve as tags, and include polypeptide sequences, such as poly-gly sequences of between about 5 and 200 amino acids. Such flexible linkers are known to persons of skill in the art. For example, poly(ethelyne glycol) linkers are available from Shearwater Polymers, Inc. Huntsville,

Alabama. These linkers optionally have amide linkages, sulfhydryl linkages, or heterofunctional linkages.

[183] Tag binders are fixed to solid substrates using any of a variety of methods currently available. Solid substrates are commonly derivatized or functionalized by exposing all or a portion of the substrate to a chemical reagent which fixes a chemical group to the surface which is reactive with a portion of the tag binder. For example, groups which are suitable for attachment to a longer chain portion would include amines, hydroxyl, thiol, and carboxyl groups. Aminoalkylsilanes and hydroxyalkylsilanes can be used to functionalize a variety of surfaces, such as glass surfaces. The construction of such solid phase biopolymer arrays is well described in the literature. *See, e.g., Merrifield, J. Am. Chem. Soc.* 85:2149-2154 (1963) (describing solid phase synthesis of, *e.g.*, peptides); Geysen *et al., J. Immun. Meth.* 102:259-274 (1987) (describing synthesis of solid phase components on pins); Frank & Doring, *Tetrahedron* 44:60316040 (1988) (describing synthesis of various peptide sequences on cellulose disks); Fodor *et al., Science*, 251:767-777 (1991); Sheldon *et al., Clinical Chemistry* 39(4):718-719 (1993); and Kozal *et al., Nature Medicine* 2(7):753759 (1996) (all describing arrays of biopolymers fixed to solid substrates). Non-chemical approaches for fixing tag binders to substrates include other common methods, such as heat, cross-linking by UV radiation, and the like.

#### 20 D. Computer-based assays

[184] Yet another assay for compounds that modulate GPCR activity involves computer assisted drug design, in which a computer system is used to generate a three-dimensional structure of GPCR based on the structural information encoded by the amino acid sequence. The input amino acid sequence interacts directly and actively with a preestablished algorithm in a computer program to yield secondary, tertiary, and quaternary structural models of the protein. The models of the protein structure are then examined to identify regions of the structure that have the ability to bind, *e.g.*, ligands. These regions are then used to identify ligands that bind to the protein.

[185] The three-dimensional structural model of the protein is generated by entering protein amino acid sequences of at least 10 amino acid residues or corresponding nucleic acid sequences encoding a GPCR polypeptide into the computer system. The amino acid sequence of the polypeptide or the nucleic acid encoding the polypeptide is selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, or 11 or SEQ ID NOs:2, 4, 6, 8, 10, or 12, respectively, and conservatively modified versions thereof. The amino acid sequence

represents the primary sequence or subsequence of the protein, which encodes the structural information of the protein. At least 10 residues of the amino acid sequence (or a nucleotide sequence encoding 10 amino acids) are entered into the computer system from computer keyboards, computer readable substrates that include, but are not limited to, electronic storage media (*e.g.*, magnetic diskettes, tapes, cartridges, and chips), optical media (*e.g.*, CD ROM), information distributed by internet sites, and by RAM. The three-dimensional structural model of the protein is then generated by the interaction of the amino acid sequence and the computer system, using software known to those of skill in the art.

[186] The amino acid sequence represents a primary structure that encodes the information necessary to form the secondary, tertiary and quaternary structure of the protein of interest. The software looks at certain parameters encoded by the primary sequence to generate the structural model. These parameters are referred to as "energy terms," and primarily include electrostatic potentials, hydrophobic potentials, solvent accessible surfaces, and hydrogen bonding. Secondary energy terms include van der Waals potentials. Biological molecules form the structures that minimize the energy terms in a cumulative fashion. The computer program is therefore using these terms encoded by the primary structure or amino acid sequence to create the secondary structural model.

[187] The tertiary structure of the protein encoded by the secondary structure is then formed on the basis of the energy terms of the secondary structure. The user at this point can enter additional variables such as whether the protein is membrane bound or soluble, its location in the body, and its cellular location, *e.g.*, cytoplasmic, surface, or nuclear. These variables along with the energy terms of the secondary structure are used to form the model of the tertiary structure. In modeling the tertiary structure, the computer program matches hydrophobic faces of secondary structure with like, and hydrophilic faces of secondary structure with like.

[188] Once the structure has been generated, potential ligand binding regions are identified by the computer system. Three-dimensional structures for potential ligands are generated by entering amino acid or nucleotide sequences or chemical formulas of compounds, as described above. The three-dimensional structure of the potential ligand is then compared to that of the GPCR protein to identify ligands that bind to GPCR. Binding affinity between the protein and ligands is determined using energy terms to determine which ligands have an enhanced probability of binding to the protein.

[189] Computer systems are also used to screen for mutations, polymorphic variants, alleles and interspecies homologs of GPCR genes. Such mutations can be associated with

disease states or genetic traits. As described above, GeneChip™ and related technology can also be used to screen for mutations, polymorphic variants, alleles and interspecies homologs. Once the variants are identified, diagnostic assays can be used to identify patients having such mutated genes. Identification of the mutated GPCR genes involves receiving input of a first nucleic acid or amino acid sequence encoding a GPCR selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, and 11, or SEQ ID Nos:2, 4, 6, 8, 10, and 12, respectively, and conservatively modified versions thereof. The sequence is entered into the computer system as described above. The first nucleic acid or amino acid sequence is then compared to a second nucleic acid or amino acid sequence that has substantial identity to the first sequence. The second sequence is entered into the computer system in the manner described above. Once the first and second sequences are compared, nucleotide or amino acid differences between the sequences are identified. Such sequences can represent allelic differences in GPCR genes, and mutations associated with disease states and genetic traits.

## Kits

[190] GPCRs and their homologs are a useful tool for identifying cells such as brain, adipose, kidney, liver, hypothalamus, colon, pancreas, skeletal muscle, heart, placenta, skin, colon, lung, thymus, prostate, stomach, small intestine, or spleen cells, for forensics and paternity determinations, for diagnosing diseases, and for examining signal transduction. GPCR specific reagents that specifically hybridize to GPCR nucleic acids, such as GPCR probes and primers, and GPCR specific reagents that specifically bind to a GPCR protein, e.g., GPCR antibodies are used to examine signal transduction regulation.

[191] Nucleic acid assays for the presence of GPCR DNA and RNA in a sample include numerous techniques are known to those skilled in the art, such as Southern analysis, northern analysis, dot blots, RNase protection, S1 analysis, amplification techniques such as PCR and LCR, and *in situ* hybridization. In *in situ* hybridization, for example, the target nucleic acid is liberated from its cellular surroundings in such as to be available for hybridization within the cell while preserving the cellular morphology for subsequent interpretation and analysis (see Example I). The following articles provide an overview of the art of *in situ* hybridization: Singer *et al.*, *Biotechniques* 4:230-250 (1986); Haase *et al.*, *Methods in Virology*, vol. VII, pp. 189-226 (1984); and *Nucleic Acid Hybridization: A Practical Approach* (Hames *et al.*, eds. 1987). In addition, GPCR protein can be detected with the various immunoassay techniques described above. The test sample is typically

compared to both a positive control (*e.g.*, a sample expressing a recombinant GPCR) and a negative control.

[192] The present invention also provides for kits for screening for modulators of GPCRs. Such kits can be prepared from readily available materials and reagents. For example, such kits can comprise any one or more of the following materials: a GPCR, reaction tubes, and instructions for testing GPCR activity. Optionally, the kit contains biologically active GPCR. A wide variety of kits and components can be prepared according to the present invention, depending upon the intended user of the kit and the particular needs of the user.

#### Disease treatment and diagnosis

[193] TGRs are involved in the regulation of many important physiological functions and are often therapeutic targets for various diseases or conditions. Mammalian TGRs are typically classified in three categories, class A, receptors related to rhodopsin and the adrenergic receptors, class B, receptors related to the calcitonin and parathyroid hormone receptors, and class C, receptors related to the metabotropic receptors. The rhodopsin/adrenergic receptor class is the largest class and includes various amine receptor, *e.g.*, acetylcholine (muscarinic) receptors, adrenergic receptors, dopamine receptors, histamine receptors, serotonin receptors, and octopamine receptors; peptide receptors, *e.g.*, angiotensin, bombesin, bradykinin, endothelin, interleukin-8, chemokine, melanocortin, neuropeptide Y, neurotensin, opioid, somatostatin, tachykinin, thrombin, vasopressin, galanin, proteinase-activated, orexin, and chemokine/chemotatic factor receptors; protein hormone receptors, *e.g.*, FSH, lutropin-choriogonadotropic hormone, and thyrotropin receptors; rhodopsin receptors; olfactory receptors; prostanoid receptors; nucleotide-like receptors, including adenosine and purinoceptors; cannabis receptors; platelet activating factor receptor; gonadotropin-releasing hormone receptor; melatonin receptor, lysosphingolipid and LPA (EDG) receptors, as well as various orphan receptors. Class B includes calcitonin, corticotropin releasing factor, gastric inhibitory peptide glucagon, growth hormone-releasing hormone, parathyroid hormone, PACAP, secretin, vasoactive intestinal polypeptide, and brain-specific angiogenesis inhibitor receptors, among others. Class C receptors include metabotropic glutamate receptors and GABA-B subtype receptors as well as putative pheromone receptors.

[194] Class A GPCRs function in a variety of physiological processes such as vasodilation, bronchodilation, neurotransmitter signaling, stimulation of endocrine secretions,



gut peristalsis, development, mitogenesis, cell proliferation, cell migration, immune system function, and oncogenesis. Accordingly, class A GPCRs can be used, for example, as probes to identify cells or tissues that exhibit dysregulation of these processes, and moreover, as screening targets to identify modulators of these processes.

5        [195] Class B GPCRs also function in a wide range of physiological processes such as regulation of calcium homeostasis, modulation of activity of cells in the immune system, various excitatory and inhibitory actions in the central nervous system, control of smooth muscle relaxation, control of smooth muscle, secretion in stomach, intestinal epithelium, pancreas, and gall bladder. Accordingly, class B GPCRs can be used, for example, as probes  
10 to identify cells or tissues that exhibit dysregulation of these process, and to identify modulators of these physiological processes.

      [196] Class C GPCRs, metabotropic glutamate receptors, are also important regulators of physiological processes such as neurotransmission. Glutamate is the major neurotransmitter in the CNS and plays an important role in neuronal plasticity, cognition,  
15 memory, learning, and some neurological disorders such as epilepsy, stroke, and neurodegeneration. B-type receptors for the neurotransmitter GABA (gamma-aminobutyric acid) inhibit neuronal activity through G-protein-coupled second-messenger systems, which regulate the release of neurotransmitters and the activity of ion channels and adenylyl cyclase. Thus, GABA B-type receptors play a role in controlling neuronal function and are  
20 also involved in such processes as neuronal plasticity, cognition, memory, and learning. Accordingly, class C GPCRs can be used, for example, as probes to identify cells or tissues, particularly, neuronal cells or tissues, that exhibit dysregulation of these processes, and to identify modulators of these physiological processes for the treatment of neurological disorders.

25        [197] In certain embodiments, the presently-described GPCRs can be used in the diagnosis and treatment of certain diseases or conditions, *i.e.*, TGR-associated disorders. For example, the activity of GPCRs (*e.g.*, TGR2, 118, 164, 358, TGR343) that are expressed or preferentially expressed in a particular cell type (*e.g.*, brain, immune tissue, or kidney), can be used to modulate cellular function (*e.g.*, responsiveness to extracellular signals), thereby  
30 specifically modulating the function of the cells of that type in a patient.

      [198] Further, mutations in the cell specific GPCRs will likely produce a disease, condition, or symptom associated with a lack of function of the particular cell type. For example, mutations in GPCRs preferentially expressed in the hypothalamus will likely result in any number of conditions associated with the hypothalamus and the pituitary gland, which

is often controlled by chemical mediators secreted by the hypothalamus. For example, dysfunction of hypothalamus-specific GPCRs can alter secretion of one or more hypothalamic factors such as growth hormone-releasing hormone, somatostatin, gonadotropin-releasing hormone, thyrotropin-releasing hormone, and corticotropin-releasing hormone. Thus, hypothalamic-associated diseases include hypothyroidism, hypogonadism, growth disorders, and hyperprolactinemia, as well as diabetes insipidus and disturbances of thirst, sleep, temperature regulation, appetite, blood pressure or any other syndrome or disease associated with the hypothalamus (*see, e.g., Harrison's Principles of Internal Medicine*, 12th Edition, Wilson, *et al.*, eds., McGraw-Hill, Inc.).

10 [199] Similarly, mutations or dysregulation of TGRs expressed in lymphocytes or hematopoietic cell-associated TGRs, *i.e.*, TGRs preferentially expressed in peripheral blood lymphocytes (PBLs), bone marrow, thymus, or hematopoietic cell lineages including cells involved in the immune system, *e.g.*, TGR2, TGR343, can lead to malignancies, anemia, and other disorders of immune function and inflammation such as asthma, inflammatory bowel syndrome, arthritis, *e.g.*, rheumatoid arthritis, multiple sclerosis, pancreatitis and other autoimmune diseases and inflammatory diseases (*see, e.g., Harrison's Principles of Internal Medicine, supra*).

[200] Disorders in the function, or level, of kidney-associated GPCRs (for example, TGR164, which is highly specific for kidney and is localized to epithelial cells of kidney tubules), will likely result in any of a number of nephrotic conditions or diseases, such as renal failure, nephritis, nephrotic syndrome, asymptomatic urinary abnormalities, renal tubule defects, hypertension, nephrolithiasis, or any other syndrome or disease associated with the kidneys (*see, e.g., Harrison's Principles of Internal Medicine*, 12th Edition, Wilson, *et al.*, eds., McGraw-Hill, Inc.). Thus, TGR164 is a target for the diagnosis of and treatment of kidney disease.

[201] Similarly, liver-associated GPCRs may play a role in liver-related disease or condition, *e.g.*, cirrhosis, infiltrations, lesions, functional disorders, and jaundice (*see, Harrison's Principles of Internal Medicine*, 12th Edition, Wilson, *et al.*, eds., McGraw-Hill, Inc.).

30 [202] Mutation or dysregulation of adipocyte GPCRs can lead to disorders relating to weight control and hyperlipidemia; and can also be used to detect, or diagnose a propensity for, conditions such as obesity. Similarly, spleen-associated GPCRs may be involved in any spleen-associated disorder or condition, *e.g.*, splenic enlargement, immune disorders, blood disorders, and others (*see, e.g., Harrison's Principles of Internal Medicine, supra*). Altered

function, or level, of GPCRs expressed in the colon can result in any of a number of colon-associated conditions or diseases, *e.g.*, inflammatory bowel disease such as Crohn's disease and ulcerative colitis, and other alterations in bowel habit, rectal bleeding, pain, and other symptoms (*see, e.g., Harrison's Principles of Internal Medicine, supra*). Mutations in GPCRs preferentially expressed in the skin can lead to any number of problems including psoriasis, lupus erythematosus, as well as other chronic inflammatory disease of the skin; acute inflammatory dermatoses, and blistering diseases. Mutations in GPCRs may also be correlated with skin malignancies and such GPCRs may play a role in infections that involve the skin (*see, e.g., Harrison's Principles of Internal Medicine, supra*) or other skin-associated diseases. Mutations or abnormal function, or level, of pancreas-associated GPCRs may play a role in diseases associated with failure to control blood glucose levels such as diabetes. Skeletal muscle-associated GPCRs may play a role in various myopathies including those that cause muscle pain; acute, subacute, or chronic muscle weakness; and other diseases of the skeletal muscle system (*see, e.g., Harrison's Principles of Internal Medicine, supra*). GPCRs expressed in the lung may be involved in disorders of pulmonary function (*see, e.g., Harrison's Principles of Internal Medicine, supra*) and GPCRs expressed in the heart may play a role in a wide variety of disorders, including heart failure, arrhythmias and various other cardiomyopathies (*see, e.g., Harrison's Principles of Internal Medicine, supra*). GPCRs expressed in the stomach and small intestine may play a role in diseases of the gastrointestinal tract (*see, e.g., Harrison's Principles of Internal Medicine, supra*).

[203] GPCRs that exhibit differential expression and/or activity during the development or progression of a disease may be useful diagnostic, prognostic, and/or therapeutic targets. Accordingly, the present sequences can be used in diagnostic applications for any of the herein-described disorders or conditions in a patient, *e.g.*, by examining the sequence, level, or activity of any of the present GPCRs in a patient; wherein an alteration, *e.g.*, a decrease or increase, in the level of expression or activity in a GPCR, or the detection of a deleterious mutation in a GPCR, indicates the presence or the likelihood of the disease or condition. Similarly, in prognostic applications, the activity or expression of a GPCR identified herein can be used as an indicator of the progression or regression of a disease. In addition, modulation of the present GPCRs (*e.g.*, by administering modulators of the GPCR) can be used to treat or prevent any of the conditions or diseases.

[204] For example, TGR2 expression is induced in the bronchoalveola during asthma development. Therefore, expression of TGR2 may be used, *e.g.*, as a diagnostic or prognostic marker. Moreover, agents that target TGR2 activity may be used to treat asthma.

[205] TGR343 is associated with immunological disorders, in particular, multiple sclerosis. Thus, TGR343 may be useful for diagnosing such immune disorders, *e.g.*, multiple sclerosis, and/or measuring the progression of the disease. Further, agents that target TGR343 activity may be used to treat multiple sclerosis and other immune disorders that involve TGR343 dysfunction.

#### Administration and pharmaceutical compositions

[206] GPCR modulators can be administered directly to the mammalian subject for modulation of signal transduction *in vivo*, *e.g.*, for the treatment of any of the diseases or conditions described *supra*. Administration is by any of the routes normally used for introducing a modulator compound into ultimate contact with the tissue to be treated. The GPCR modulators are administered in any suitable manner, optionally with pharmaceutically acceptable carriers. Suitable methods of administering such modulators are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

[207] Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present invention (*see, e.g., Remington's Pharmaceutical Sciences*, 17<sup>th</sup> ed. 1985)).

[208] The GPCR modulators, alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[209] Formulations suitable for administration include aqueous and non-aqueous solutions, isotonic sterile solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions can be administered, for example, orally, topically, intravenously, intraperitoneally, intravesically or intrathecally. Optionally, the compositions are administered nasally. The formulations of compounds can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials. Solutions and suspensions can be prepared from sterile powders, granules, and tablets of the

kind previously described. The modulators can also be administered as part of a prepared food or drug.

[210] The dose administered to a patient, in the context of the present invention, should be sufficient to effect a beneficial response in the subject over time. Such doses are administered prophylactically or to an individual already suffering from the disease. The compositions are administered to a patient in an amount sufficient to elicit an effective protective or therapeutic response in the patient. An amount adequate to accomplish this is defined as "therapeutically effective dose." The dose will be determined by the efficacy of the particular GPCR modulators (e.g., GPCR antagonists and anti-GPCR antibodies) employed and the condition of the subject, as well as the body weight or surface area of the area to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular compound or vector in a particular subject.

[211] In determining the effective amount of the modulator to be administered in a physician may evaluate circulating plasma levels of the modulator, modulator toxicities, and the production of anti-modulator antibodies. In general, the dose equivalent of a modulator is from about 1 ng/kg to 10 mg/kg for a typical subject.

[212] For administration, GPCR modulators of the present invention can be administered at a rate determined by the LD-50 of the modulator, and the side-effects of the inhibitor at various concentrations, as applied to the mass and overall health of the subject. Administration can be accomplished via single or divided doses.

[213] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

#### EXAMPLES

[214] The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

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**I. Example I: Identification of novel GPCRs**

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[215] Six novel GPCRs were identified by searching the public databases (Swiss-Prot and Genbank) and their nucleic acid sequences are provided in SEQ ID NOs:1, 3, 5, 7, 9, and 11. The deduced amino acid sequences are provided in SEQ ID NOs:2, 4, 6, 8, 10, and 12. The novel GPCRs were designated TGR2, TGR38, TRG118, TGR164, TGR343, and TGR358.

**TGR2**

[216] TGR2 was identified from genomic sequences by searching the public databases. It is available under the accession number AP000808. TGR2 is 31% identical to the mouse orphan GPR90 and 29% identical to the rat MAS1 oncogene.

[217] Expression of TGR2 was analyzed in human tissues by expression profiling using PCR. RT-PCR was performed using the following primer set: forward primer, 5' AAGGTCCACGAGCTGATGAAGA 3'; reverse primer, 5' AACAGATGAGGAACAC CAGGAC 3'. One microgram of total RNA from each tissue was reverse transcribed and then used as a template in a PCR reaction of 35 cycles, each of which was: 94°C, 30 sec; 60°C, 1 min; and 72°C, 2 min. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control. The results show that TGR2 is expressed in brain, adipose, kidney, skeletal muscle, and placenta. TGR2 is also highly expressed in immune tissues including lymph node and thymus in both mouse (*see, e.g.*, Figure 1) and human. Moreover, TGR2 expression is induced in a mouse model of asthma, particularly in bronchoalveolar lavage samples (Figure 2).

**TGR38**

[218] TGR38 was identified from genomic sequences by searching the public databases. It is available under the accession number AC021016 (Genbank) and is 28% identical to human EDG6.

[219] Expression of TGR38 was analyzed in human tissues by expression profiling using PCR. RT-PCR was performed using the following primer set: forward primer, 5' ACTTCTCCTTCCTCTCCCTGCT 3'; and reverse primer, 5' GACACTGCTTTGG CTGCTTG 3'. One microgram of total RNA from each tissue was reverse transcribed and then used as a template in a PCR reaction of 35 cycles, each of which was: 94°C, 30 sec; 60°C, 1 min; and 72°C, 2 min. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was

used as an internal control.. The results showed that a TGR38 mRNA is expressed in adipose, placenta, kidney, pancreas, spleen, skin, colon, skeletal muscle, liver, and brain.

#### TGR118

[220] TGR118 was also identified from genomic sequences by searching the public databases. It is 25% identical to rabbit serotonin receptor 1D beta.

[221] Expression of TGR118 was analyzed in human tissues by expression profiling using PCR. RT-PCR was performed using the following primer set: forward primer, 5' GGCCTACATTCTCCTCCACA 3'; reverse primer, 5' CATGGGACATGAAGGAGAGG 3'.

One microgram of total RNA from each tissue was reverse transcribed and then used as a template in a PCR reaction of 35 cycles, each of which was: 94°C, 30 sec; 60°C, 1 min; and 72°C, 2 min. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control. The results showed that TGR118 mRNA is expressed in the brain and peripheral blood lymphocytes (PBLs).

#### TGR164

[222] TGR164 was also identified from genomic sequences by searching the public databases. It is available under the accession number AC060810 (Genbank). TGR164 is 35% identical to human P2Y1.

[223] Expression of TGR164 was analyzed in human tissues by expression profiling using PCR. RT-PCR was performed using the following primer set: forward primer, 5' AAAATGAGACCTTGAAGAGCA 3'; reverse primer, 5' CCTCAAGATATGGAAGGG TAAAAA 3'. One microgram of total RNA from each tissue was reverse transcribed and then used as a template in a PCR reaction of 35 cycles, each of which was: 94°C, 30 sec; 60°C, 1 min; and 72°C, 2 min. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control. The results showed TGR164 mRNA was expressed in brain, kidney, adipose, pancreas, lung, and colon. In particular, TGR164 was highly expressed in the kidney, relative to other tissues. Further evaluation of expression using *in situ* hybridization shows that TGR164 is localized to the epithelial cells of the kidney tubules.

#### TGR343

[224] TGR343 was also identified from genomic sequences by searching the public databases. It is available under the accession number AC083865 (Genbank). TGR343 is 21% identical to rat CysLT1 receptor.

[225] Expression of TGR343 was analyzed in human tissues by expression profiling using PCR. RT-PCR was performed using the following primer set: forward primer, 5' ACCCCACTTAATCAGCCTCTAC 3'; reverse primer, 5' CAGTGCTCCTCATTTGTA TTCCTC 3'. One microgram of total RNA from each tissue was reverse transcribed and then used as a template in a PCR reaction of 35 cycles, each of which was: 94°C, 30 sec; 60°C, 1 min; and 72°C, 2 min. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control. The results showed that TGR 343 mRNA is expressed in hypothalamus, adipose, liver, kidney, placenta, lung, colon, thymus, spleen, and skin. In particular, TGR343 is highly expressed in immune tissues including bone marrow and spleen (*see, e.g.* Figure 3).

[226] TGR343 was also shown to be induced in multiple sclerosis using a mouse model of multiple sclerosis. Samples from experimental autoimmune encephalomyelitis (EAE) animals (*Nat. Rev Neurosci* 3:291-301, 2002) exhibited markedly increased expression of TGR343 in comparison to controls (Figure 4).

#### TGR358

[227] TGR358 was also identified from genomic sequences by searching the public databases. It is available under the accession number chrX 0360 (Genbank). TGR358 shares 24% sequence identity with human orphan receptor GPR34.

[228] Expression of TGR358 was analyzed in human tissues by expression profiling using PCR. RT-PCR was performed using the following primer set: forward primer, 5' CATCAACGCACATCTACCTGTCAC 3'; reverse primer, 5' CTGAGAGATCATGGCTC CTAGTTCC 3'. One microgram of total RNA from each tissue was reverse transcribed and then used as a template in a PCR reaction of 35 cycles, each of which was: 94°C, 30 sec; 60°C, 1 min; and 72°C, 2 min. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control. The results demonstrated that TGR358 mRNA was expressed in several various tissues including brain, adipose, skeletal muscle, heart, placenta, colon, prostate, stomach, small intestine, kidney, lung, thymus, and spleen. Higher levels of expression were typically observed in the immune system.

[229] All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.



## SEQUENCES

**SEQ ID NO:1** Human TGR2 Nucleic Acid

The underlined ATG indicates the start methionine.

- 5 ATGAACCAGACTTTGAATAGCAGTGGGACCGTGGAGTCAGCCCTAAACTATTCCAGAGGGAG  
CACAGTGCACACGGCCTACCTGGTGCTGAGCTCCCTGGCCATGTTACCTGCCTGTGCGGGA  
TGGCAGGCAACAGCATGGTGATCTGGCTGCTGGGCTTTCGAATGCACAGGAACCCCTTCTGC  
ATCTATATCCTCAACCTGGCGGCAGCCGACCTCCTCTTCTCCTCCTCAGCATGGCTTCCACGCT  
CAGCCTGGAAACCCAGCCCCTGGTCAATACCACTGACAAGGTCCACGAGCTGATGAAGAGAC  
10 TGATGTACTTTGCCTACACAGTGGGCCTGAGCCTGCTGACGGCCATCAGCACCCAGCGCTGT  
CTCTCTGTCTCTTCCCTATCTGGTTC AAGTGTACCGGCC CAGGCACCTGTCAGCCTGGGT  
GTGTGGCCTGCTGTGGACACTCTGTCTCCTGATGAACGGGTGACCTCTTCCTTCTGCAGCA  
AGTTCTTGAAATTCAATGAAGATCGGTGCTTCAGGGTGGACATGGTCCAGGCCGCCCTCATC  
ATGGGGGTCTTAACCCAGTGATGACTCTGTCCAGCCTGACCCTCTTTGTCTGGGTGCGGAG  
15 GAGCTCCCAGCAGTGGCGGCGGCAGCCACACGGCTGTTCTGTGGTGGTCCTGGCCTCTGTCC  
TGGTGTCTCTCATCTGTTCCCTGCCTCTGAGCATCTACTGGTTTGTGCTCTACTGGTTGAGC  
CTGCCGCCCCGAGATGCAGGTCTGTGCTTCAGCTTGTCACGCCTCTCCTCGTCCGTAAGCAG  
CAGCGCCAACCCCGTCATCTACTTCCTGGTGGGCAGCCGGAGGAGCCACAGGCTGCCACCA  
GGTCCCTGGGGACTGTGCTCCAACAGGCGCTTCGCGAGGAGCCCGAGCTGGAAGGTGGGGAG  
20 ACGCCACCGTGGGCACCAATGAGATGGGGGCT

**SEQ ID NO:2** Human TGR2 Protein sequence

- MNQTLNSSGTVESALNYSRGSTVHTAYLVLSSLAMFTCLCGMAGNSMVIWLLGFRMHRNPFC  
IYIILNLAAADLLFLFSMASTLSLETQPLVNTTDKVHELMKRLMYFAYTVGLSLLTAISTQRC  
25 LSVLFPFIWFKCHRPRHLSAWVCGLLWTLCLLMNGLTSSFCSKFLKFNEDRCFRVDMVQAALI  
MGVLTPVMTLSSLTLFVWVRRSSQWRRQPTRLFVVVLASVLVFLICSLPLSIYWFVLYWLS  
LPPEMQVLCFSL SRLSSSVSSSANPVIYFLVGSRRSHRLPTRSLGTVLQQALREEPELEGGE  
TPTVGTNEMGA

- 30 **SEQ ID NO:3** Human TGR38 Nucleic Acid sequence

The underline indicates the position of the start methionine.

GAGCCTGGCCCCGCTGTCCCCACTGGGTGGAGACACCATGCACCTGGTCCACTTGTGCTCTT  
CAGCCAGGACACCAGACATGGTCCAAACCGCTGCAGGGCTGGCTGCAGCAACTCCCTGACAC

TCAGGAAGGCCAGGCTGGGCAGGCAATACCTGCTCCCAACAGCCATGCATGCCGGCTGCCG  
 CTCCAGGACTCCCCTGTCCCCAGGACCAAGATGACGCCCAACAGCACTGGCGAGGTGCCAG  
 CCCCATTCCCAAGGGGGCTTTGGGGCTCTCCCTGGCCCTGGCAAGCCTCATCATCACCGCGA  
 ACCTGCTCCTAGCCCTGGGCATCGCCTGGGACCGCCGCTGCGCAGCCACCTGCTGGCTGC  
 5 TTCTTCCTGAGCCTACTGCTGGCTGGGCTGCTCACGGGTCTGGCATTGCCACATTGCCAGG  
 GCTGTGGAACCAGAGTCGCCGGGGTTACTGGTCCTGCCTCCTCGTCTACTTGGCTCCCAACT  
 TCTCCTTCCTCTCCCTGCTTGCCAACCTCTTGCTGGTGCACGGGGAGCGCTACATGGCAGTC  
 CTGAGGCCACTCCAGCCCCCTGGGAGCATTCCGGCTGGCCCTGCTCCTCACCTGGGCTGGTCC  
 CCTGCTCTTTGCCAGTCTGCCCCGCTCTGGGGTGGAAACCACTGGACCCCTGGTGCCAACTGCA  
 10 GCTCCCAGGCTATCTTCCCAGCCCCCTACCTGTACCTCGAAGTCTATGGGCTCCTGCTGCCC  
 GCCGTGGGTGCTGCTGCCCTTCTCTGTCCGCGTGCTGGCCACTGCCACCGCCAGCTGCA  
 GGACATCTGCCGGCTGGAGCGGGCAGTGTGCCGCGATGAGCCCTCCGCCCTGGCCCGGGCCC  
 TTACCTGGAGGCAGGCAAGGGCACAGGCTGGAGCCATGCTGCTCTTCGGGCTGTGCTGGGGG  
 CCCTACGTGGCCACACTGCTCCTCTCAGTCCTGGCCTATGAGCAGCGCCCGCCACTGGGGCC  
 15 TGGGACACTGTTGTCCCTCCTCTCCCTAGGAAGTGCCAGTGCAGCGGCAGTGCCCGTAGCCA  
 TGGGGCTGGGCGATCAGCGCTACACAGCCCCCTGGAGGGCAGCCGCCCAAAGGTGCCTGCAG  
 GGGCTGTGGGGAAGAGCCTCCCGGGACAGTCCCGGCCCCAGCATTGCCTACCACCCAAGCAG  
 CCAAAGCAGTGTGACCTGGACTTGAATAAAGGAAGGGCCTCTGCTGACTCCTACCAGAGC  
 ATCCGTCCAGCTCAGCCATCCAGCCTGTCTCTACTGGGCCCCACTTCTCTGGATCAAGACCC  
 20 TGCTCTGTTTGACCCCGCACTGACTGAATAAAGCTCCTCTGGCCGTTAAAAAAAAAAAAA

**SEQ ID NO:4** Human TGR38 Protein Sequence

MTPNSTGEVPSPIPKGALGLSLALASLIITANLLLALGIAWDRRLRSPPAGCFFLSLLLAGL  
 LTGLALPTLPGLWNQSRGYWSCLLVYLAPNFSFLSLLANLLLHGERYMAVLRPLQPPGSI  
 25 RLALLLTWAGPLLFPALGWNHWTPGANCSSQAIFPAPYLYLEVYGLLLPAVGAAAFLSV  
 RVLATAHRQLQDICRLERAVCRDEPSALARALTWRQARAQAGAMLLFGLCWGPYVATLLLSV  
 LAYEQRPPPLPGTLLSLLSLGSASAAVPVAMGLGDQRYTAPWRAAQRCLQGLWGRASRDS  
 PGPSIAYHPSSQSSVDLDLN

**SEQ ID NO:5** Human TGR118 Nucleic Acid

The underlined ATG indicates the start methionine.

ATGGGGGATGAGCTGGCACCTTGCCCTGTGGGCACTACAGCTTGGCCGGCCCTGATCCAGCT  
 CATCAGCAAGACACCTGCATGCCCCAAGCAGCCAGCAACACTTCCTTGGGCCTGGGGGACC  
 TCAGGGTGCCAGCTCCATGCTGTACTGGCTTTTCCTTCCCTCAAGCCTGCTGGCTGCAGCC

ACACTGGCTGTCAGCCCCCTGCTGCTGGTGACCATCCTGCGGAACCAACGGCTGCGACAGGA  
GCCCCACTACCTGCTCCCGGCTAACATCCTGCTCTCAGACCTGGCCTACATTCTCCTCCACA  
TGCTCATCTCCTCCAGCAGCCTGGGTGGCTGGGAGCTGGGCCGCATGGCCTGTGGCATTCTC  
ACTGATGCTGTCTTCGCCGCTGCACCAGCACCATCCTGTCCTTCACCGCCATTGTGCTGCA  
5 CACCTACCTGGCAGTCATCCATCCACTGCGCTACCTCTCCTTCATGTCCCATGGGGCTGCCT  
GGAAGGCGATGGCCCTCATCTGGCTGGTGGCCTGCTGCTTCCCCACATTCCCTTATTTGGCTC  
AGCAAGTGGCAGGATGCCCAGCTGGAGGAGCAAGGAGCTTCATACATCCTACCACCAAGCAT  
GGGCACCCAGCCGGGATGTGGCCTCCTGGTCATTGTTACCTACACCTCCATTCTGTGCGTTC  
TGTTCCCTCTGCACAGCTCTCATTGCCAACTGTTTCTGGAGGATCTATGCAGAGGCCAAGACT  
10 TCAGGCATCTGGGGGCAGGGCTATTCCCGGGCCAGGGGCACCCTGCTGATCCACTCAGTGCT  
GATCACATTGTACGTGAGCACAGGGGTGGTGTCTCCCTGGACATGGTGCTGACCAGGTACC  
ACCACATTGACTCTGGGACTCACACATGGCTCCTGGCAGCTAACAGTGAGGTA CT CATGATG  
CTTCCCCGTGCCATGCTCCCATACCTGTACCTGCTCCGCTACCGGCAGCTGTTGGGCATGGT  
CCGGGGCCACCTCCCATCCAGGAGGCACCAGGCCATCTTTACCATTTCCTAG

15

**SEQ ID NO:6** Human TGR118 Protein

MGDELAPCPVGT TAWPALIQLISKTPCMPQAASNTSLGLGDLRVPSSMLYWLFLPSSLLAA  
TLAVSPLLLVTILRNQRLRQEPHYLLPANILLSDLAYILLHMLISSSSLGGWELGRMACGIL  
TDAVF AACTSTILSFTAIVLHTYLAVIHPLRYLSFMSHGAAWKAVALIWLVACCFPTFLIWL  
20 SKWQDAQLEEQGASYILPPSMGTQPGCGLLVIVTYTSILCVLFLCTALIANCFWRIYAEAKT  
SGIWGQGYSRARGTLLIHSVLI TLYVSTGVVFS LDMVLTRYHHIDSGTHTWLLAANSEVLMM  
LPRAMLTLYLLRYRQLLGMVRGHLPSRRHQAI FTIS

**SEQ ID NO:7** Human TGR164 Nucleic Acid

25 The underlined ATG indicates the start methionine.

ATGAATGAGCCACTAGACTATTTAGCAAATGCTTCTGATTTCCTCCGATTATGCAGCTGCTTT  
TGGA AATTGCACTGATGAAACATCCCACTCAAGATGCACTACCTCCCTGTTATTTATGGCA  
TTATCTTCCTCGTGGGATTTCCAGGCAATGCAGTAGTGATATCCACTTACATTTTCAA AATG  
AGACCTTGGAAGAGCAGCACCATCATTATGCTGAACCTGGCCTGCACAGATCTGCTGTATCT  
30 GACCAGCCTCCCCTTCCTGATTCACTACTATGCCAGTGGCGAA AACTGGATCTTTGGAGATT  
TCATGTGTAAGTTTATCCGCTTCAGCTTCCATTTCAACCTGTATAGCAGCATCCTCTTCCTC  
ACCTGTTTCAGCATCTTCCGCTACTGTGTGATCATTACCCAATGAGCTGCTTTTCCATTCA  
CAAACTCGATGTGCAGTTGTAGCCTGTGCTGTGGTGTGGATCATTTCAGTGGTAGCTGTCA  
TTCCGATGACCTTCTTGATCACATCAACCAACAGGACCAACAGATCAGCCTGTCTCGACCTC

ACCAGTTCGGATGAACTCAATACTATTAAGTGGTACAACCTGATTTTGACTGCAACTACTTT  
CTGCCTCCCCTTGGTGATAGTGACACTTTGCTATACCACGATTATCCACACTCTGACCCATG  
GACTGCAAACTGACAGCTGCCTTAAGCAGAAAGCACGAAGGCTAACCATTCTGCTACTCCTT  
GCATTTTACGTATGTTTTTTACCCTTCCATATCTTGAGGGTCATTCCGATCGAATCTCGCCT  
5 GCTTTCAATCAGTTGTTCCATTGAGAATCAGATCCATGAAGCTTACATCGTTTCTAGACCAT  
TAGCTGCTCTGAACACCTTTGGTAACCTGTTACTATATGTGGTGGTCAGCGACAACCTTTCAG  
CAGGCTGTCTGCTCAACAGTGAGATGCAAAGTAAGCGGGAACCTTGAGCAAGCAAAGAAAAT  
TAGTTACTCAAACAACCCTTGA

10 **SEQ ID NO:8** Human TGR164 Protein

MNEPLDYLANASDFPDYAAAFGNCTDENIPLKMHYLPVIYGIIFLVGFPGNAVVISITYIFKM  
RPWKSSTIIMLNLA CTDLLYLTSLPFLIHYYASGENWIFGDFMCKFIRFSFHFNLSSILFL  
TCFSIFRYCVIIHPMSCFSIHKTRCAVVACAVVWIIISLVAVIPMTFLITSTNRTNRSACLDL  
TSSDELNTIKWYNLILTATTFCLPLVIVTLCYTTIIHTLTHGLQTDSCCLKQKARRLTILLLL  
15 AFYVCFLPFHILRVIRIESRLLSISCS IENQIHEAYIVSRPLAALNTFGNLLLVVVSDNFQ  
QAVCSTVRCKVSGNLEQAKKISYSNNP

**SEQ ID NO:9** Human TGR343 Nucleic Acid

ATGCTGGCCACAATACCTCCAGGAATTCCTCTTGCGATCCTATAGTGACACCCCACTTAAT  
20 CAGCCTCTACTTCATAGTGCTTATTGGCGGGCTGGTGGGTGTCATTTCCATTCTTTTCCTCC  
TGGTGAAAATGAACACCCGGTCAGTGACCACCATGGCGGTCATTA ACTTGGTGGTGGTCCAC  
AGCGTTTTTTCTGCTGACAGTGCCATTTGCTTGACCTACCTCATCAAGAAGACTTGGATGTT  
TGGGCTGCCCTTCTGCAAATTTGTGAGTGCCATGCTGCACATCCACATGTACCTCACGTTCC  
TATTCTATGTGGTGATCCTGGTCACCAGATACCTCATCTTCTTCAAGTGCAAAGACAAAGTG  
25 GAATTCTACAGAAAAC TGATGCTGTGGCTGCCAGTGCTGGCATGTGGACGCTGGTGATTGT  
CATTGTGGTACCCCTGGTTGTCTCCCGGTATGGAATCATGAGGAATACAATGAGGAGCACT  
GTTTTAAATTTACAAAGAGCTTGCTTACACATATGTGAAAATCATCAACTATATGATAGTC  
ATTTTTGT CATAGCCGTTGCTGTGATTCTGTTGGTCTTCCAGGTCTTCATCATTATGTTGAT  
GGTGCAGAAGCTACGCCACTCTTTACTATCCCACCAGGAGTTCTGGGCTCAGCTGAAAAACC  
30 TATTTTTTATAGGGGTCATCCTTGTTTGTTCCTTCCCTACCAGTTCTTTAGGATCTATTAC  
TTGAATGTTGTGACGCATTCCAATGCCTGTAAACAGCAAGGTTGCATTTTATAACGAAATCTT  
CTTGAGTGTAACAGCAATTAGCTGCTATGATTGCTTCTCTTGTCTTTGGGGGAAGCCATT  
GGTTTAAGCAAAAGATAATTGGCTTATGGAATTGTGTTTTGTGCCGTTAG

**SEQ ID NO:10** Human TGR343 Protein

MPGHNTSRNSSCDPIVTPHLISLYFIVLIGGLVGVISILFLLVKMNTRSVTTMAVINLVVVH  
SVFLLTVPFRLTYLIKKTWMFGLPFCKFVSAMLIHMYLTFLFYVVILVTRYLIFFKCKDKV  
EFYRKLHAVAASAGMWTLVIVIVVPLVVSRYGIHBEYNEEHCFKFHKELAYTYVKIINYMIV  
5 IFVIAVAVILLVFQVFIIMLMVQKLRSLLSHQEFWAQLKNLFFIGVILVCFLPYQFFRIYY  
LNVVTHSNACNSKVAFYNEIFLSVTAISCYDLLLFVFGGSHWFKQKIIGLWNCVLCR

**SEQ ID NO:11** Human TGR358 Nucleic Acid

ATGAACAACAATACAACATGTATTCAACCATCTATGATCTCTTCCATGGCTTTACCAATCAT  
10 TTACATCCTCCTTTGTATTGTTGGTGTGTTTTGGAAACACTCTCTCTCAATGGATATTTTAA  
CAAAAATAGGTAAAAAACATCAACGCACATCTACCTGTACACCTTGTGACTGCAAACTTA  
CTTGTGTGCAGTGCCATGCCTTTCATGAGTATCTATTTCTGAAAGGTTTCCAATGGGAATA  
TCAATCTGCTCAATGCAGAGTGGTCAATTTCTGGGAAGTCTATCCATGCATGCAAGTATGT  
TTGTCAGTCTCTTAATTTTAAGTTGGATTGCCATAAGCCGCTATGCTACCTTAATGCAAAAG  
15 GATTCCTCGCAAGAGACTACTTCATGCTATGAGAAAATATTTTATGGCCATTTACTGAAAA  
ATTCGCCAGCCCAACTTTGCTAGAAACTATGCATTTACATATGGGGAGTTGTACTGGGCA  
TAATCATTCAGTTACCGTATACTACTCAGTCATAGAGGCTACAGAAGGAGAAGAGAGCCTA  
TGCTACAATCGGCAGATGGAAGTAGGAGCCATGATCTCTCAGATTGCAGGTCTCATTGGAAC  
CACATTTATTGGATTTTCCTTTTGTAGTAGTACTAACATCATACTACTCTTTTGTAAAGCCATC  
20 TGAGAAAAATAAGAACCTGTACGTCCATTATGGAGAAAGATTGACTTACAGTTCTGTGAAA  
AGACATCTTTTGGTCATCCAGATTCTACTAATAGTTTGCTTCCTTCCTTATAGTATTTTAA  
ACCCATTTTTTATGTTCTACACCAAAGAGATAACTGTCAGCAATTGAATTATTTAATAGAAA  
CAAAAACATTCTCACCTGTCTTGCTTCGGCCAGAAGTAGCACAGACCCCATATATTTCTT  
TTATTAGATAAAACATTCAAGAAGACACTATATAATCTCTTTACAAAGCTAATTCAGCACA  
25 TATGCAATCATATGGTTGA

**SEQ ID NO:12** Human TGR358 Protein

MNNNTTCIQPSMISSMALPIIYILLCIVGVFGNTLSQWIFLTKIGKKTSTHIYLSHLVTANL  
LVCSAMPFMSIYFLKGFQWEYQSAQCRVVNFLGTLMSHASMFVSLILLSWIAISRYATLMQK  
30 DSSQETTSCYEKIFYGHLKKFRQPNFARKLCIYIWGVVLGIIIPVTVYYSVIEATEGEESL  
CYNRQMELGAMISQIAGLIGTTFIGFSFLVVLTSYYSFVSHLRKIRTCTSIMKDLTYSSVK  
RHLLVIQILLIVCFLPYSIFKPIFYVLHQRDNCQQLNYLIETKNILTCLASARSSTDPIIFL  
LLDKTFKKTLYNLFTKSNSAHMQSYG

WHAT IS CLAIMED IS:

1                   1.     An isolated nucleic acid encoding a polypeptide comprising greater  
2     than 70% amino acid sequence identity to an amino acid sequence of SEQ ID NO:2, SEQ ID  
3     NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12

1                   2..    The isolated nucleic acid of claim 1, wherein the polypeptide  
2     comprises greater than 80% amino acid sequence identity to an amino acid sequence of SEQ  
3     ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

1                   3.     The isolated nucleic acid of claim 1, wherein the polypeptide  
2     comprises greater than 90% amino acid sequence identity to an amino acid sequence of SEQ  
3     ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

1                   4.     The isolated nucleic acid of claim 1, wherein the nucleic acid  
2     encodes a polypeptide that specifically binds to polyclonal antibodies generated against  
3     an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8,  
4     SEQ ID NO:10, or SEQ ID NO:12.

1                   5.     The isolated nucleic acid of claim 1, wherein the nucleic acid  
2     encodes a polypeptide that has G-protein coupled receptor activity.

1                   6.     The isolated nucleic acid of claim 1, wherein the nucleic acid  
2     encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:2, SEQ ID  
3     NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10; or SEQ ID NO:12.

1                   7.     The isolated nucleic acid of claim 1, wherein the nucleic acid  
2     comprises the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ  
3     ID NO:7, SEQ ID NO:9, or SEQ ID NO:11.

1                   8.     The isolated nucleic acid of claim 1, wherein the nucleic acid is  
2     amplified by primers that specifically hybridize under stringent hybridization conditions  
3     to a nucleic acid comprising the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3,  
4     SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11.

1                   9.     An isolated nucleic acid, wherein the nucleic acid specifically  
2     hybridizes under stringent hybridization conditions to a nucleic acid comprising the

3 nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ  
4 ID NO:9, or SEQ ID NO:11.

1 10. An isolated nucleic acid encoding a polypeptide comprising greater  
2 than 70% amino acid identity to a polypeptide comprising an amino acid sequence of  
3 SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID  
4 NO:12, wherein the nucleic acid selectively hybridizes under moderately stringent  
5 hybridization conditions to the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3,  
6 SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11.

1 11. An isolated nucleic acid encoding a polypeptide comprising at least  
2 20 contiguous amino acids of an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4,  
3 SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

1 12. An isolated polypeptide comprising greater than 70% amino acid  
2 sequence identity to an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID  
3 NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

1 13. The isolated polypeptide of claim 12, wherein the polypeptide  
2 comprises greater than 80% identity to an amino acid sequence of SEQ ID NO:2, SEQ ID  
3 NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

1 14. The isolated polypeptide of claim 12, wherein the polypeptide  
2 comprises greater than 90% identity to an amino acid sequence of SEQ ID NO:2, SEQ ID  
3 NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

1 15. The isolated polypeptide of claim 12, wherein the polypeptide has  
2 G-protein coupled receptor activity.

1 16. The isolated polypeptide of claim 12, wherein the polypeptide  
2 specifically binds to polyclonal antibodies generated against SEQ ID NO:2, SEQ ID  
3 NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

1 17. The isolated polypeptide of claim 12, wherein the polypeptide  
2 comprises the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ  
3 ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

- 1                   18.    An isolated polypeptide comprising at least 20 contiguous amino  
2   acids of an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID  
3   NO:8, SEQ ID NO:10, or SEQ ID NO:12.
- 1                   19.    An antibody that selectively binds to the polypeptide of claim 12 or  
2   claim 18.
- 1                   20.    An expression vector comprising the nucleic acid of claim 1, claim  
2   9, or claim 11.
- 1                   21.    A host cell transfected with the vector of claim 20.
- 1                   22.    A method for identifying a compound that modulates signal  
2   transduction, the method comprising:  
3                   (i) contacting the compound with a polypeptide comprising greater than  
4   70% amino acid sequence identity to an amino acid sequence of SEQ ID NO:2, SEQ ID  
5   NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12; and  
6                   (ii) determining the functional effect of the compound upon the  
7   polypeptide.
- 1                   23.    A method for identifying a compound that modulates signal  
2   transduction, the method comprising:  
3                   (i) contacting the compound with a polypeptide comprising at least 20  
4   contiguous amino acids of an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ  
5   ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12; and  
6                   (ii) determining the functional effect of the compound upon the  
7   polypeptide.
- 1                   24.    The method of claim 22, wherein the polypeptide comprises greater  
2   than 80% amino acid sequence identity to an amino acid sequence of SEQ ID NO:2, SEQ  
3   ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.
- 1                   25.    The method of claim 22, wherein the polypeptide comprises greater  
2   than 90% amino acid sequence identity to an amino acid sequence of SEQ ID NO:2, SEQ  
3   ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.



1                   26.    The method of claim 22 or claim 23, wherein the polypeptide has  
2    G-protein coupled receptor activity.

1                   27.    The method of claim 22 or claim 23, wherein the polypeptide is  
2    linked to a solid phase.

1                   28.    The method of claim 22 or claim 23, wherein the functional effect  
2    is determined by measuring changes in intracellular cAMP, IP<sub>3</sub>, or Ca<sup>2+</sup>.

1                   29.    The method of claim 22 or claim 23, wherein the functional effect  
2    is a chemical effect.

1                   30.    The method of claim 22 or claim 23, wherein the functional effect  
2    is a physical effect.

1                   31.    The method of claim 22 or claim 23, wherein the functional effect  
2    is determined by measuring binding of the compound to the polypeptide.

1                   32.    The method of claim 22 or claim 23, wherein the polypeptide is  
2    recombinant.

1                   33.    The method of claim 22 or claim 23, wherein the polypeptide  
2    comprises the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ  
3    ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

1                   34.    The method of claim 22 or claim 23, wherein the polypeptide is  
2    expressed in a cell or cell membrane.

1                   35.    The method of claim 22 or claim 23, wherein the cell is a  
2    eukaryotic cell.

1                   36.    A method of identifying a mammal having a TGR-associated  
2    disorder, the method comprising detecting a nucleic acid molecule that comprises greater  
3    than 70% nucleic acid sequence identity to a sequence of SEQ ID NO:1, SEQ ID NO:3,  
4    SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11 in a sample from the  
5    mammal, wherein abnormal expression of the nucleic acid molecule in the sample  
6    indicates that the mammal has a TGR-associated disorder.

1                   37.    The method of claim 36, wherein the nucleic acid molecule  
2   comprises the sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7,  
3   SEQ ID NO:9, or SEQ ID NO:11.

1                   38.    A method of identifying a mammal having a TGR-associated  
2   disorder, the method comprising detecting a nucleic acid molecule that comprises at least  
3   20 contiguous nucleotides of a nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:3,  
4   SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11 in a sample from the  
5   mammal, wherein abnormal expression of the nucleic acid molecule in the sample  
6   indicates that the mammal has a TGR-associated disorder.

1                   39.    A method of identifying a mammal having a TGR-associated  
2   disorder, the method comprising detecting a polypeptide that comprises greater than 70%  
3   amino acid sequence identity to an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4,  
4   SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12 in a sample from the  
5   mammal, wherein abnormal expression of the polypeptide in the sample indicates that the  
6   mammal has a TGR-associated disorder.

1                   40.    The method of claim 39, wherein the polypeptide comprises the  
2   amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8,  
3   SEQ ID NO:10, or SEQ ID NO:12.

1                   41.    A method of identifying a mammal having a TGR-associated disorder,  
2   the method comprising detecting a polypeptide that comprises at least 20 contiguous amino  
3   acids of an amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID  
4   NO:8, SEQ ID NO:10, or SEQ ID NO:12 in a sample from the mammal, wherein abnormal  
5   expression of the polypeptide in the sample indicates that the mammal has a TGR-associated  
6   disorder.

1                   42.    A method of treating or preventing a TGR-associated disorder, the  
2   method comprising administering a therapeutically effective amount of a compound  
3   identified using the method of claim 22 or claim 23 to a mammal in need thereof.

1                   43.    The method of claim 42, wherein the method comprises administering  
2   a TGR2 modulator to a patient who has, or is at risk for, is asthma.

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1                   44.    The method of claim 42, wherein the method comprises administering  
2   a TGR343 modulator to a patient who has, or is at risk for, multiple sclerosis.

1                   45.    The method of claim 42, wherein the method comprises administering  
2   a TGR164 modulator to a patient who has, or is at risk for, kidney disease.

Fig. 1

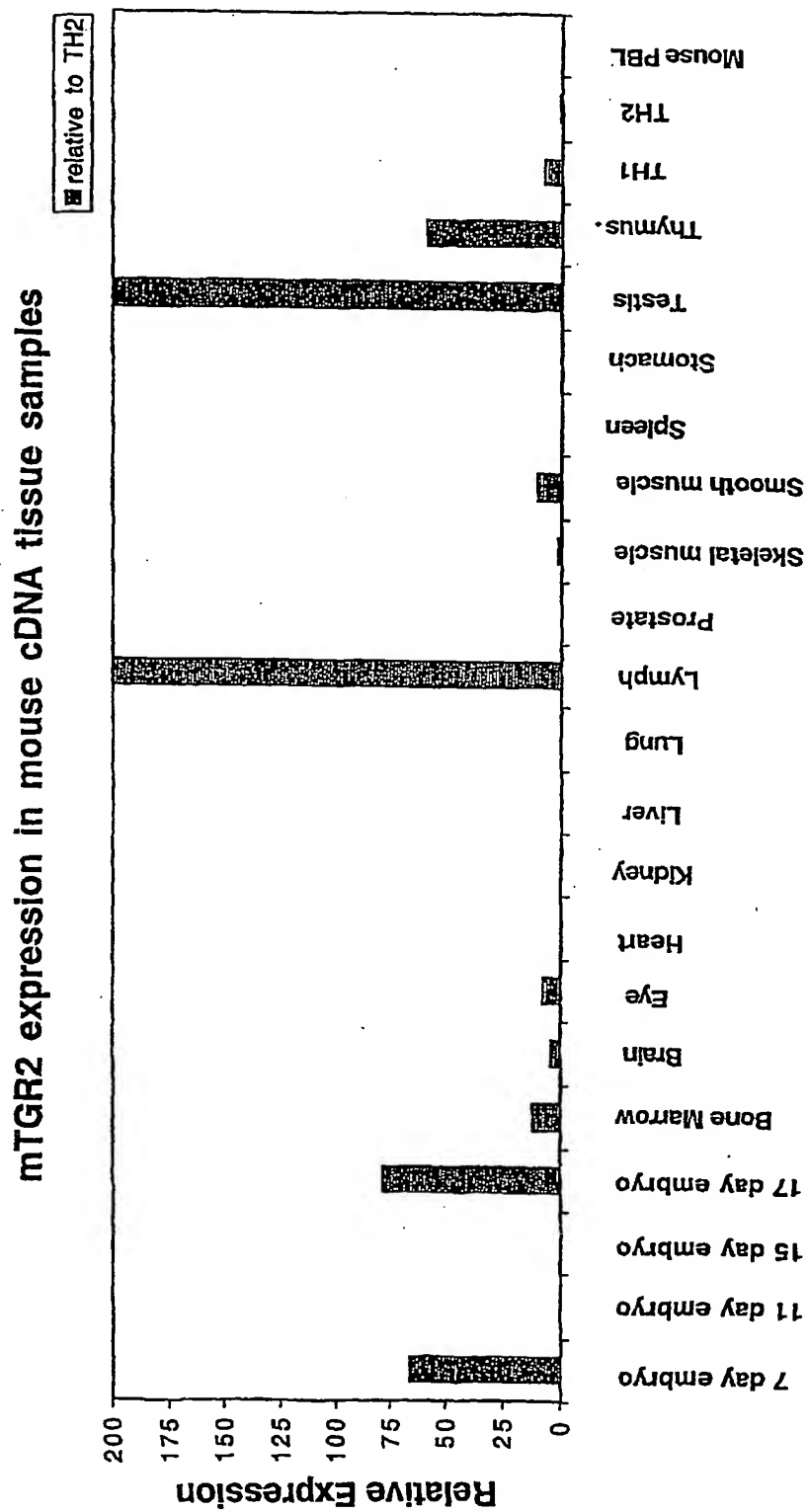
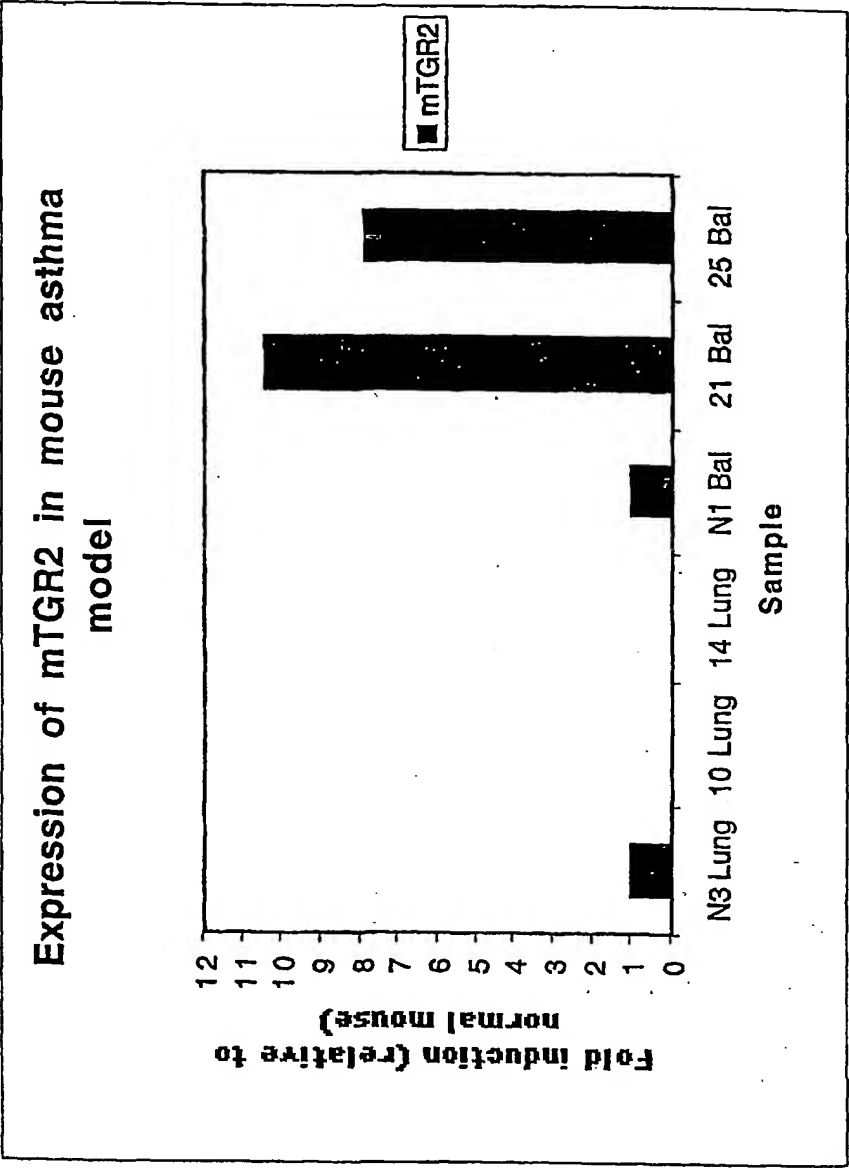


Fig. 2



BAL: bronchoalveolar lavage

Fig. 3

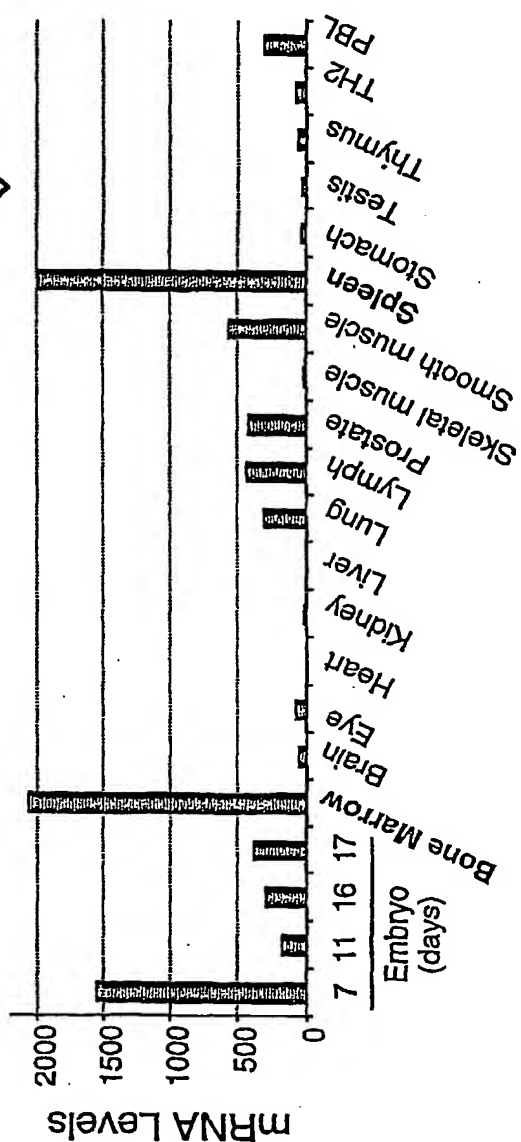
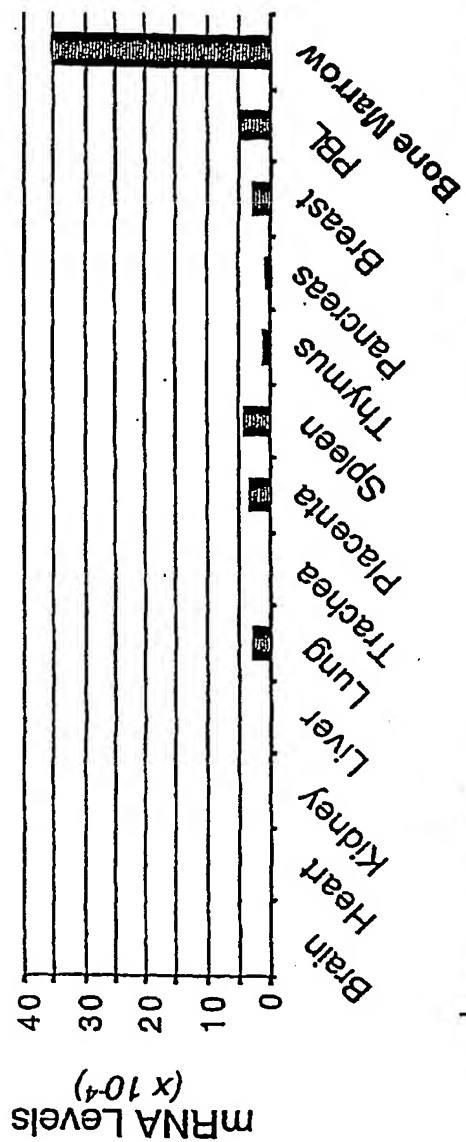


Fig. 4

➤ Expression of mTGR343 and a positive control gene in mouse EAE samples

